Hyaluronan:RHAMM Mediated Signal Transduction: Functions in Focal Adhesion Turnover, Cell Locomotion and Tumorigenesis

By

Christine L. Hall

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Physiology
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

COPYRIGHT PERMISSION

HYALURONAN: RHAMM MEDIATED SIGNAL TRANSDUCTION: FUNCTIONS IN FOCAL ADHESION TURNOVER, CELL LOCOMOTION AND TUMORIGENESIS

by

CHRISTINE L. HALL

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Abstract

Extracellular matrix (ECM) molecules and their receptors are important regulators of adhesion, migration, cytoskeletal organization, gene expression, growth and differentiation. Moreover, ECM receptors can serve to mediate signal transduction pathways that can influence, and sometimes direct, the events required for tumorigenesis. In particular, the extracellular matrix molecule hyaluronan (HA) has been associated with tumorigenesis, tumor cell migration, invasion and metastasis. Through the study of HA receptors, such as CD44 and RHAMM, the relationship between HA and tumor progression has changed from correlative to causative. This thesis addresses the role of one of these receptors, RHAMM (Receptor for HA Mediated Motility), in the regulation tumor cell locomotion and tumorigenesis. By examining the signal transduction pathways and cytoskeletal alterations involved in HA:RHAMM induced cell locomotion, this study has identified tyrosine phosphorylation within focal adhesions as a key event in the HA:RHAMM cell motility pathway; it has revealed a role for RHAMM in tumorigenesis; it has provided a functional link between RHAMM and two oncogene products, *ras* and *src*; and it has predicted that tyrosine phosphorylation and focal adhesion turnover play important roles in both cell locomotion and tumorigenesis. The results here are presented as a series of three papers: Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity, *J. Cell Biol.*, 126:575-588, 1994; Overexpression of the hyaluronan receptor RHAMM is transforming and is also required for H-*ras* transformation, *Cell*, 82:19-28, 1995;

and pp60$^{c\text{-}src}$ is required for cell locomotion regulated by the hyaluronan receptor RHAMM, *Oncogene*, in press, 1996. In combination, these results suggest a model in which HA and RHAMM are central players in the regulation of the actin cycle, cell motility and transformation.

Acknowledgments

The journey I began in the fall of 1990 was a long and winding one. Like all journeys, new friends were encountered and some old ones came along for the ride, new places were explored, new things were learned and the old knowledge was reinforced, many obstacles were overcome but some backtracking was required, and in the end there was relief the trip was over, but gratification of the journey taken. When I became a graduate student 6 years ago I was Christine, now I have become Chris, much the same but eyes more open, both looking out and looking in.

A rocky road always seems smoother when there is a good navigator. I would like to thank Eva Turley for being that navigator. Like every good captain, she directed me along the journey, yet she often let me guide my own way. In addition, I could have never completed this journey without the several pit stops I made along the way. Thank-you to my inspirational pit crew, my committee, Vetta Kardami, John Wilkins and David Litchfield. A special thanks to my external examiner Ivan Stamenkovic.

Those of you who took the ride with me know the journey all to well. Thank-you to my co-Turley girls (especially Laurie, Carol and Gen), my companions in the Turley lab and my friends in the department of Cell Biology and Physiology for making my ride smoother. I will not refer to everyone by name (they know who they are)- but one spiritual advisor can not go unmentioned. I am but one of the feeders of VAL.

Finally, I would like to thank the one that I came home to every night when the day was done, my husband, my life long companion, my lover, my friend - David Minor. Thank-you for holding me up and standing by my side.

Table of Contents

List of Figures and Tables

Introduction and Literature Review

Chapters: Experimentation, Results and Discussion

Chapter 1: Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity

List of Abbreviations

aa	amino acids
Ab	antibody
αMEM	minimal essential media
bp	base pairs
BSA	bovine serum albumin
CD44s	standard or hematopoietic form of CD44
CD44v	variant form of CD44
DMEM	Dulbecco's modified Eagle Medium
ECM	extracellular matrix
EGF	epidermal growth factor

ERM	ezrin/radixin/moesin protein family
FACS	fluorescence-activated cell sorter
FAK	focal adhesion kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FGF	fibroblast growth factor
GPI	glycosylphosphatidylinositol
h	hour(s)
HA	hyaluronan
HBSS	Hank's balanced salt solution
HSPGs	heparan sulfate proteoglycans
IGF	insulin-like growth factor
kb	kilobases
LPA	lysophosphatidic acid
MAb	monoclonal antibody
MAP kinase	mitogen activated protein kinase
MEK	MAP/ERK kinase
min	minute(s)
ng	nanograms
NGF	nerve growth factor
PBS	phosphate buffered saline
PCR	polymerize chain reaction
PDGF	platelet-derived growth factor
PIP_2	phosphatidylinositol 4,5-diphosphate
PKC	protein kinase C
PTK	protein tyrosine kinase
RHAMM	receptor for HA mediated cell motility
RIPA	radio-immunoprecipitation assay
RT-PCR	reverse transcription PCR

s	second(s)
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	mean standard error
SH2	src homology 2 domain
SH3	src homology 3 domain
SS	signal sequence
TBS	tris buffered saline
TGF-β	transforming growth factor-beta
TIMPs	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
TPA	tetradecanoylphorbol acetate
TRITC	tetramethylrhodamine isothiocyanate
TSG-6	TNF-stimulated gene-6 product
TTBS	Tween-20 TBS
uPAR	urokinase plasminogen activator receptor
VASP	vasodilator-stimulated phosphoprotein

Introduction

and

Literature Review

Introduction

Tumorigenesis has long been associated with the unrestrained growth of cells, and the metastatic spread of tumors has been linked to the ability of these cells to locomote and invade. Many cellular proteins control cell growth, and hence tumorigenesis, by signaling changes in gene expression, growth, cell cycling and invasion (see Bishop, 1991). Among these proteins are the extracellular matrix (ECM) molecules and their receptors, which have been shown to regulate tumor progression and metastasis (Starkey, 1990; Aznavoorian *et al.*, 1993; Giancotti & Mainiero, 1994). In particular, the integrin family of ECM receptors (see Hynes, 1992) and the hyaluronan (HA) receptors (see Sherman *et al.*, 1994) are important regulators of tumorigenesis, tumor progression, invasion and metastasis (Turley *et al.*, 1987; Gehlsen *et al.*, 1988; Humphries *et al.*, 1988; McCarthy *et al.*, 1988; Giancotti & Ruoslahti, 1990; Reber *et al.*, 1990; Chan *et al.*, 1991; Gunthert *et al.*, 1991; Schreiner *et al.*, 1991; Sy *et al.*, 1991 & 1992; Felding-Habermann *et al.*, 1992; Seftor *et al.*, 1992; Rudy *et al.*, 1993; Seiter *et al.*, 1993; Qian *et al.*, 1994; Hall *et al.*, 1995). These ECM receptors elicit many of their effects on tumor cell function by triggering signal transduction pathways. Indeed, ECM molecules can stimulate or modulate the same signaling pathways induced by growth factors and neuropeptides (see Clark & Brugge, 1995; Rozengurt, 1995). These pathways involve the activation/expression of cellular oncogenes. In particular, the integrin mediated signal transduction pathways have been shown to be important for adhesion, migration, growth and gene expression, (see Hynes, 1992; Juliano & Haskill, 1993; Giancotti

& Mainiero, 1994; Schwartz & Ingber, 1994; Clark & Brugge, 1995; Schwartz *et al.*, 1995). Likewise, the hyaluronan receptors are emerging as key regulators of adhesion, cellular signaling, cell motility and growth, and hence tumorigenesis and metastasis (see Sherman *et al.*, 1994).

There is a strong connection between the ECM carbohydrate hyaluronan and the processes of transformation and metastasis (Turley, 1984; Laurant & Fraser, 1992; Knudson & Knudson, 1993). HA is often found to be enriched in tumor associated stroma (Knudson *et al.*, 1989) and the presence of HA has been linked to increased cell locomotion, an essential property of invading tumor cells (see Zetter, 1990). HA has been found to stimulate the invasion of tumor cells (Turley *et al.*, 1987; Wang & Turley, 1994), and the HA receptors CD44 and RHAMM have been linked to tumor cell locomotion (Hart *et al.*, 1991; Turley *et al.*, 1991; Hardwick *et al.*, 1992; Thomas *et al.*, 1992; Hall *et al.*, 1994 & 1995). As well, highly metastatic tumor cells produce more HA and express higher levels of HA receptors than their non-metastatic counterparts (Knudson *et al.*, 1989; Turley, 1992; Gunthert, 1993; Knudson & Knudson, 1993). Recently, through the study of HA receptors, the connection between HA and tumorigenesis has changed from correlative to causative. Exemplary to this statement is the work by Dr. Turley's group on the role of the HA receptor, RHAMM (Receptor for HA Mediated cell Motility) in tumor cell locomotion and tumorigenesis. This thesis addresses some of the molecular mechanisms behind HA:RHAMM induced tumor cell locomotion and begins to define the role of this receptor in tumorigenesis and metastasis.

The following sections are designed to introduce the role of the extracellular matrix in tumorigenesis and to address how ECM signaling affects adhesion, cell motility and other cell characteristics. In each section, special attention will be paid to hyaluronan and the hyaluronan receptors. In addition to this literature review, the reader should refer to the prefaces and introductions in Chapters 1-3 in the following Experimentation, Results and Discussion section.

The Extracellular Matrix: Architecture, Adhesion and Signaling

The extracellular matrix - definition: The extracellular matrix, as the term implies, is the network of macromolecules filling the non-cellular portion of tissues. Traditionally, the ECM was defined as an assembly of cell-derived structural components comprised of collagens, non-collagenous glycoproteins (e.g. laminin, fibronectin, tenascin), proteoglycans (e.g. aggrecan, perlecan, decorin), glycosaminoglycans (e.g. hyaluronan) and elastin (Schuppan & Hahn, 1987). More recently the definition of ECM has been expanded to include molecules that are biologically or functionally associated with these structural components including growth factors [e.g. fibroblast growth factor (FGF), transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF)], matrix degrading proteases and their inhibitors [e.g. metalloproteinases and tissue inhibitors of metalloproteinases (TIMPs)], and ECM receptors (e.g. integrins, heparan sulfate proteoglycans, HA receptors; Schuppan *et al.*, 1994). The ECM can perform both a structural role and a functional role in the regulation of cell shape, differentiation state and proliferation.

ECM receptors - a brief overview: ECM receptors are generally categorized according to their structural homology. As a result, cell-matrix and cell-cell receptors are often clustered together under the title of adhesion molecules or adhesion receptors. Adhesion receptors are divided into four major super-families, the cadherins, the immunoglobulin super-family of adhesion receptors, the selectins and the integrins (Hynes, 1994; Rosales *et al.*, 1995). Many of these receptors are primarily involved in cell-cell adhesion (for a review of these receptors and their signaling capabilities, the reader should refer to Juliano & Haskill, 1993; Rosales *et al.*, 1995; Gumbiner, 1996). Here, I will discuss only those receptors involved in cell-matrix adhesion.

The integrin family of receptors can engage in both cell-cell and cell-matrix adhesion. These ECM receptors are heterodimeric glycoproteins comprised of two integral membrane subunits, an α and a β subunit. The combination of at least 15 α subunits with 8 β subunits produces 21 or more distinct integrin receptors, each of which have distinct binding properties (Hynes, 1992). The extracellular domains of the two integrin subunits interact to form a specific ligand binding pocket which may bind to cell adhesion molecules on the cell surface of adjacent cells, or to ECM ligands, including fibronectin, collagen, laminin, vitronectin, thrombin, fibrinogen, thrombospondin, and osteopontin (Hynes, 1992). The cytoplasmic domains of both the α and β subunits can serve to anchor these protein to the cytoskeleton and to transmit intracellular signals (see the focal adhesions section below).

While integrins are often referred to as the classic or prototypic ECM

receptors, additional families of ECM receptors include some of the adhesion receptors mentioned above, the HA receptors (Sherman *et al.*, 1994; Entwistle *et al.*, 1996) and the integral membrane heparan sulfate proteoglycans (HSPGs; Bernfield *et al.*, 1992; David, 1993), as well as additional receptors that are yet to be structurally defined (Buck & Horwitz, 1987). The membrane-associated HSPGs include the syndecan-related HSPGs, which are transmembrane receptors and the glypican-related HSPGs, which are linked to the cell surface via a glycosyl phosphatidylinositol (GPI) tail (David, 1993). The syndecans have highly sulfated extracellular domains that interact with the heparin/heparan sulfate binding domains of ECM matrix components, growth factors and extracellular enzymes and their inhibitors and an intracellular domain which may attach to the cytoskeleton. The hyaluronan receptors are described below.

The hyaluronan receptors: The hyaluronan binding proteins, or hyaladherins, have been classified into two groups, 1) the ECM proteins that bind to hyaluronan, including link protein, aggrecan and versican and 2) the cell-associated hyaluronan receptors, including CD44, RHAMM, and TSG-6 (Knudson & Knudson, 1993; Nelson *et al.*, 1995). While all of the HA binding proteins in group 1 share structural homology, CD44 and RHAMM, in group 2 are predominantly unrelated in their structure, although they do share a common HA binding motif[1]. This minimum HA binding consensus sequence is defined as BX_7B, where B is either lysine or arginine

[1]Note: other sequences within HA receptors also contribute HA binding (e.g. Peach *et al.*, 1993).

and X is any amino acid except aspartic acid and glutamic acid (Yang *et al.*, 1994). RHAMM and CD44 have two of these domains in their extracellular regions (Yang *et al.*, 1994) and TSG-6 contains one BX_7B motif (Nelson *et al.*, 1995). Outside of this binding motif, CD44 and TSG-6 are also structurally related with both showing 30-40% identity to their ECM counterparts, cartilage link protein and aggrecan.

CD44, also referred to as gp85, Pgp-1, ECMRIII or the Hermes antigen, was the first of the HA receptors to be identified (Underhill & Toole, 1979; Underhill *et al.*, 1987; Aruffo *et al.*, 1990). The most common form of CD44, CD44s (hematopoietic or standard form), is a broadly distributed transmembrane glycoprotein consisting of an extensively glycosylated (N- and O-linked) 37 kDa protein core that occurs as a 85-90 kDa cell surface protein. In addition, several alternatively spliced variant forms of CD44, or CD44v, are produced from the splicing of 10 additional exons and occur with a more restricted pattern of expression, most notably in malignant cells (see Sherman *et al.*, 1994). The extracellular domain of CD44 recognizes other ligands in addition to HA including fibronectin and collagen (Carter & Wayner, 1988; Jalkanen & Jalkanen, 1992), mucosal addressin (Picker *et al.*, 1989), chondroitin sulfate (Naujokas *et al.*, 1993), and the high molecular weight proteoglycan, serglycin (Toyama-Sorimachi *et al.*, 1995). The cytoplasmic domain of CD44 interacts with the actin cytoskeleton (Lacy & Underhill, 1987) specifically with the protein ankyrin (Bourguignon *et al.*, 1991) and the ezrin/radixin/moesin (ERM) family (Tsukita *et al.*, 1994). Cytoskeletal interactions may be key to CD44 function as post-translational modifications of the

CD44 cytoplasmic domain, including serine phosphorylation (Kalomiris & Bourguignon, 1989), palmitylation (Bourguignon *et al.*, 1991), and GTP binding (Lokeshwar & Bourguignon, 1992) have been reported to affect its cytoskeletal associations. Furthermore, cytoskeletal associations and truncation or modification of the cytoplasmic domain of CD44 can interfere with HA binding (Thomas *et al.*, 1992; He *et al.*, 1992; Lesley & Hyman, 1992; Liao *et al.*, 1993; Lokeshwar *et al.*, 1994). CD44 has many functions including a role in lymphocyte activation (Haynes *et al.*, 1989), lymphocyte homing (Jalkanen *et al.*, 1987), HA internalization and degradation (Culty *et al.*, 1992; Hua *et al.*, 1993), cell-cell (St. John *et al.*, 1990) and cell-ECM adhesion (Aruffo *et al.*, 1990; Underhill, 1992; Thomas *et al.*, 1992; Lesley *et al.*, 1994) and cell migration (Hart *et al.*, 1991; Thomas *et al.*, 1992). Several of these functions depend on the HA binding capacity of CD44.

RHAMM, the second HA receptor to be characterized, has emerged as a critical regulator of cell motility, cytoskeletal structure and cell proliferation (Hardwick *et al.*, 1992; Turley, 1992; Entwistle *et al.*, 1996). RHAMM is broadly expressed *in vitro* in both normal cells, such as fibroblasts, smooth muscle cells, white blood cells, neural cells and sperm, and in malignant cells, including fibrosarcoma, multiple myeloma B cells, small cell lung carcinomas and breast carcinoma cells (Boudreau *et al.*, 1991; Turley *et al.*, 1991, 1993 & 1994; Hardwick *et al.*, 1992; Kornovski *et al.*, 1994; Pilarski *et al*, 1993 & 1994; Hall & Turley, 1995; Nagy *et al.*, 1995; Savani & Turley, 1995; Teder *et al.*, 1995; Entwistle *et al.*, 1996; Masellis-Smith *et al.*, 1996). Physiologically, RHAMM has been shown to be

important in the cell locomotion processes of wound repair (Savani *et al.*, 1995), restenosis (Savani & Turley, 1995) and development (Boudreau *et al.*, 1991). Pathologically, RHAMM is required for tumor cell locomotion and proliferation, and hence may be critical for tumorigenesis, invasion and metastasis (see Hall & Turley, 1995).

Protein expression of RHAMM is low in quiescent cells but becomes markedly increased during cell migration, following cytokine stimulation and in transformed cells (Hardwick *et al.*, 1992; Samuel *et al.*, 1993). The murine RHAMM gene is located on mouse chromosome 11 and is comprised of 14 exons, 3 of which have already been shown to be alternatively spliced (Entwistle *et al.*, 1995; Spicer *et al.*, 1995). All of the encoded RHAMM proteins have two HA binding domains located in the carboxy-terminal region (Yang *et al.*, 1993) and several potential sites for N-glycosylation (Entwistle *et al.*, 1995; Hardwick *et al.*, 1992). Like CD44, RHAMM has a potential for alternative splicing and post-translational modification that give rise to numerous tissue and species specific protein isoforms. These include both soluble and membrane forms of RHAMM with a range in molecular size from 52 to 120 kDa (see Figure 1). In addition to the originally isolated RHAMM2 (Hardwick *et al.*, 1992), the 70 kDa RHAMM1 is the most commonly expressed form of RHAMM in non-transformed murine tissue (Entwistle *et al.*, 1995). Furthermore, a 73 kDa variant (RHAMM1v4) is detected at very low levels, however its expression is markedly elevated in transformed cells (Entwistle *et al.*, 1995). Often a 100-110 kDa form of RHAMM is detected and may

represent another glycosylation or splice variant (unpublished data). As well, the human RHAMM gene has been mapped to chromosome 5q33.2-qter (Spicer *et al.*, 1995). One human RHAMM isoform has been isolated from a human breast cDNA expression library and found to be 85% homologous to murine RHAMM with the HA binding domains 100% conserved (Wang *et al.*, 1996). The expressed RHAMM proteins are detected on the cell surface, and found to accumulate in the ECM and in the cytoplasm.

Interestingly, RHAMM belongs to a group of cell surface receptors that possess neither a strong signal peptide nor a transmembrane domain. Other receptors with these characteristics include the high affinity elastin/laminin receptors and certain animal lectins (Entwistle *et al*, 1996). It has been suggested that these proteins are transported to the surface by other carrier proteins and associate with the cell surface via integral docking proteins (Turley *et al.*, 1992). One possible mechanism for RHAMM docking is in a cluster of proteins associated with HA synthase (Klewes *et al.*, 1993). Alternatively, at least one isoform of RHAMM is inserted into the membrane via a glycosyl phosphatidylinositol (GPI) tail (Klewes *et al.*, manuscript in preparation) and this may be the primary mechanism of membrane association for this HA receptor. The urokinase plasminogen activator receptor (uPAR) is an example of another GPI-linked protein (Ploug *et al.*, 1991). uPAR is able to interact with the cytoskeleton at focal adhesions via interactions with integrins (Wei *et al.*, 1996). Furthermore, these uPAR - integrin interactions alter adhesive interactions (Wei *et al.*, 1996). Similarly, the means by which RHAMM associates

with the cell surface has very important implications for RHAMM:cytoskeletal associations and RHAMM mediated signal transduction. Several membrane proteins that are peripheral or modified with glycolipid have been reported to signal and associate, albeit indirectly, with the cytoskeleton (Resh, 1993; Anderson, 1994). Like these proteins, RHAMM is localized to focal adhesions and HA:RHAMM induced signaling results in cytoskeletal alterations (see the Experimentation, Results and Discussion section of this thesis).

TSG-6, or TNF-Stimulated Gene-6 product, is a secreted HA binding protein that has been found to be associated with inflammation, particularly arthritis (Lee *et al.*, 1992 & 1993). Because TSG-6 is not a matrix protein, it has been grouped with the cell-surface hyaluronan receptors, although the TSG-6 cDNA encodes a 29 kDa core protein without a transmembrane domain. TSG-6 may be a soluble HA binding protein or it may be membrane associated like RHAMM. Further work is required to show the association of TSG-6 with the cell membrane, if any, and to determine whether TSG-6 acts as a cell surface HA receptor. The implicated role of TSG-6 in the inflammation of arthritis makes this molecule an interesting candidate for future studies.

Focal adhesions - physical connections between the ECM and the cytoskeleton; functional sites for ECM-signaling: The above ECM receptors often cluster together to form sites of receptor-matrix adhesion. Focal adhesions, or focal contacts, are the sites of these interactions, and are so named for their dense focal appearance when examined by interference reflection microscopy. At focal

adhesions, transmembrane receptors anchor the cell to the ECM by attaching extracellularly to the matrix and intracellularly to the cytoskeletal proteins (Figure 2; Burridge *et al.*, 1988; Woods & Couchman, 1988; Luna & Hitt, 1992; Lo & Chen, 1994; Jockusch *et al.*, 1995). In culture, epithelial, endothelial and fibroblastic cells will form focal adhesions when they attach to an ECM substratum, while *in vivo*, focal adhesions structures can form between epithelial cells and the basement membrane and between migrating cells and exposed ECM during embryogenesis and wound repair. During cell movement focal adhesions are constantly being formed as the cell moves forward, and disassembled at the rear of the cell as the cell detaches. Likewise, when a cell divides, adhesions are released, then formed again as daughter cells move apart.

At focal adhesions, cytoskeletal proteins assemble in an orderly and distinct fashion to yield a tight network of actin filaments and associated proteins clustered at sites of ECM attachment (Figure 2). The dynamics of focal adhesion formation and disassembly, the defined protein-protein interactions at the cytoplasmic and extracellular faces of focal adhesions, and the regulation of these interactions have been extensively studied and reviewed (Burridge *et al.*, 1988; Woods & Couchman, 1988; Luna & Hitt, 1992; Lo & Chen, 1994; Jockusch *et al.*, 1995; Opus, 1995; Schwartz *et al.*, 1995; Craig & Johnson, 1996; Zigmond, 1996). In these studies, the integrins (primarily β1, but also β2, β3 and β5) have proven to be fundamental to focal adhesion formation. Actin filaments become anchored to the cytoplasmic tail of the β1 integrin subunit via α-actinin, which is an actin-cross-linking protein, and

by talin, which functions to nucleate, cap and cross-link actin filaments. Vinculin and tensin in turn bind to actin filaments, α-actinin, talin and each other. Other cytoskeletal proteins, such as paxillin, tenuin, filamin, the ERM proteins, zyxin, cortactin, VASP (vasodilator-stimulated phosphoprotein) and profilin, make up the remaining structural components of focal adhesions (see Figure 2). In addition to integrins, other ECM receptors interact with the cytoskeleton at focal adhesions. For example, the ERM actin capping proteins bind to CD44 (Tsukita *et al.*, 1994) perhaps acting to anchor actin filaments at focal adhesions. Syndecan-4 is also concentrated at sites of focal adhesions, where it may act as a co-receptor with integrins for the ECM (Woods & Couchman, 1994). Furthermore, RHAMM was recently identified within focal adhesions where it may influence these structures during cell motility (unpublished data). Thus, the role of the ECM receptors in focal adhesions is functional as well as structural.

In recent years, focal adhesions have been shown to be sites of multiple signal transduction pathways which, not only influence cytoskeletal assembly, but also regulate growth and differentiation (Juliano & Haskill, 1993; Zachary & Rozengurt, 1992; Lo & Chen, 1994; Clark & Brugge, 1995; Schwartz *et al.*, 1995; Craig & Johnson, 1996; Zigmond, 1996). A diverse group of regulatory molecules have been identified within focal adhesions or have been found to be associated with focal adhesion proteins. These signaling molecules include protein kinase C (PKC; Jaken *et al.*, 1989; Woods & Couchman, 1992), tyrosine kinase oncoproteins (Rohrschneider, 1980; Rohrschneider & Najita, 1984), the focal adhesion kinase,

FAK (Hanks *et al.*, 1992; Schaller *et al.*, 1992), the cellular src family kinases (Cobb *et al.*, 1994; Schaller *et al.*, 1994), csk (Bergman *et al.*, 1995), SH2-SH3 adapter proteins, crk, Grb-2, sos (Birge *et al.*, 1993; Schlaepfer *et al.*, 1994), the small GTP binding protein rho A (Zachary & Rozengurt, 1992), heterotrimeric G proteins (Hansen *et al.*, 1994) and Cdc2 (Lo & Chen, 1994). These signaling molecules form distinct pathways that can be regulated by ECM:receptor interactions. For instance, integrins activate MEK and MAP kinase which can regulate gene expression and mitogenesis (Kapron-Bras *et al.*, 1993; Chen *et al.*, 1994; Schlaepfer *et al.*, 1994; Marshall, 1995; Craig & Johnson, 1996; Chen *et al.*, 1996). ECM pathways to Cdc2 could potentially regulate cell cycle progression (Nurse, 1990; Lo & Chen, 1994). And small GTP binding proteins and G proteins can regulate the release of second messengers or lipid metabolites (Jamney, 1994; Neer, 1995; Schafer & Cooper, 1995; Zigmond, 1996).

In addition to signaling proteins, many of the structural components of focal adhesions contain phosphorylation sites as well as specific protein-, calcium- and lipid-binding motifs that can be regulated by signal transduction. By targeting cytoskeletal proteins, signal transduction within focal adhesions can influence cytoskeletal assembly/disassembly, adhesion and cell motility. For example, in response to ECM:integrin interactions, tyrosine kinases, like src, csk, and FAK, may phosphorylate cytoskeletal proteins. Likewise, activation of the serine/threonine kinase, PKC, may result in cytoskeletal phosphorylation or may trigger lipid hydrolysis that can affect actin assembly (Clark & Brugge, 1995; Schwartz et al.,

1995). Furthermore, extracellular ligand interactions with ECM receptors, growth factor receptors or neuropeptide receptors at distant cellular sites can result in the translocation of signaling molecules to focal adhesions. The result is a complex network of signals coming together at focal adhesion sites. These signaling networks are addressed further below and in the following Chapters and Summary/Model sections.

Of the signals initiated at focal adhesions, tyrosine phosphorylation has been shown to be one of the most critical for focal adhesion assembly (Guan *et al.*, 1991; Kornberg *et al.*, 1991; Burridge *et al.*, 1992). Tyrosine kinases such as abl, src family kinases and FAK are localized to focal adhesions where they may phosphorylate focal adhesion proteins and influence protein-protein interactions. For example, paxillin phosphorylation can result in paxillin-crk interactions (Birge *et al.*, 1993) via SH2 domain-phosphotyrosine interactions[2]. Of the tyrosine kinases in focal adhesions, FAK has been shown to be a key component in the signaling by both integrins and growth factors/neuropeptides (Burridge *et al.*, 1992; Zachary & Rozengurt, 1992; Parsons *et al.*, 1994; Schaller & Parsons, 1994; Clark & Brugge, 1995; Richardson & Parsons, 1995; Rozengurt, 1995). Indeed, FAK phosphorylation and kinase activity is associated with focal adhesion assembly and integrin signaling, while dephosphorylation of FAK has been often associated with disassembly. In

[2]SH2 (src homology 2) domains are found in many regulatory and some cytoskeletal proteins (e.g. tensin) and can interact with specific sequences containing phosphotyrosine (Anderson *et al.*, 1990; Matsuda *et al.*, 1990; Moran *et al.*, 1990; Songyang *et al.*, 1993; Cantley & Songyang, 1994; reviewed by Cantley *et al.*, 1991; Marengere & Pawson, 1994).

addition to functioning in cytoskeletal assembly/disassembly, FAK is a central component of other pathways at focal adhesions (e.g. FAK forms complexes with c-src, Grb-2 and Sos; Schlaepfer *et al.*, 1994). Many models hypothesize that FAK is the hub of the signal transduction unit at focal adhesions, and that ECM signaling through FAK can alter cell adhesion, cell motility and cell growth.

In addition to tyrosine phosphorylation, other post-translational modifications and high affinity interactions with Ca^{2+}, Ca^{2+}/calmodulin or polyphosphoinositide lipids can result in the refolding of proteins. Just a slight change in protein structure can result in the exposure of protein-interactive domains, e.g. SH3 (src homology 3, proline binding domains), LIM domains (cysteine-rich structural motifs which form two zinc fingers), PH (pleckstrin homology) domains (Cohen *et al.*, 1995). Alternatively, changes in protein structure can render the protein available for further post-translation modification. At focal adhesions such restructuring can alter the cytoskeletal assembly or adhesion to the ECM (see Lo & Chen, 1994; Jockusch *et al.*, 1995; Craig & Johnson, 1996; Gumbiner, 1996).

Focal adhesions and cell motility: The dynamic properties of focal adhesions are very important for cell locomotion. Cell motility results from a complex cycle of adhesion/de-adhesion; cytoskeletal assembly/disassembly; membrane ruffling and pseudopod extension at the cell front; and release and retraction at the rear of the cell. Many of the adhesive interactions and cytoskeletal rearrangements involved in cell movement originate at focal adhesions from the signaling pathways engaged there. The remaining actin cytoskeleton changes appear to originate from other signaling

pathways activated elsewhere along the cytoskeletal-plasma membrane interface (Luna & Hitt, 1992). The initiation of cell locomotion, usually the result of an extracellular stimulus, involves membrane protrusion at the leading edge, or the directional front of the cell (Stossel, 1993; Huttenlocher *et al.*, 1995; Mitchison & Cramer, 1996). A motility response to a gradient of motility factor (chemokine) results in directional membrane protrusion at the site of the highest chemokine concentration. The movement of a cell along such a concentration gradient is referred to as chemotaxis, if the chemokine is soluble, or haptotaxis, if the factor is attached to the substratum. In addition, random cell locomotion (chemokinesis) occurs in response to a number of motogens (motility factors). In this situation, no gradient is required and directional membrane protrusion appears to be random.

During membrane protrusion, actin filaments polymerize and cross-link to form a cytoskeletal lattice that supports the growing lamellipodia, or pseudopod. Within the pseudopod, actin filament cross-linking, capping, severing and attachment to the membrane are tightly controlled by changes in local calcium concentrations, polyphosphoinositide levels and protein phosphorylation (Jamney *et al.*, 1994; Jockusch *et al.*, 1995). At the base of the extending pseudopod, focal adhesions form and serve as sites for the generation of traction for movement and sites of signaling (Huttenlocher *et al.*, 1995; Mitchison & Cramer, 1996). On the upper surface of the extension, membrane ruffles appear like small waves moving toward the rear of the cell (Ridley, 1994). Although membrane ruffles are part of the motility response, their biological function is not yet known. It is hypothesized that they may be

residual waves of actin polymerization, important sites for nutrient uptake by pinocytosis or alternatively, sites for internalization of receptors and associated signal molecules that are important for cell locomotion (Ridley, 1994). While forward membrane protrusion is occurring, cell adhesions at the rear of the cell are being released for detachment (Huttenlocher *et al.*, 1995). All of these stages must occur in a constant cycle of events for the continuation of cell locomotion. While clues are being gathered on how the individual steps of membrane protrusion, focal adhesion assembly, rear detachment and contractile forces occur, the larger mystery, of how all of these events are regulated to result in cell motility, remains unsolved.

In many of the above events, the small GTPases in the Rho family, Rac, Rho and Cdc42, appear to play a large role. For instance, activated Cdc42 induces filopodia formation (long narrow membrane protrusions), activated Rho induces stress fiber formation and activated Rac induces membrane ruffles and is involved in lamellipodia formation (Nobes & Hall, 1995). Although identification of the particular pathways involved in GTPase-induced cytoskeletal changes are still under investigation, it is presumed that downstream signaling events lead to actin polymerization and hence, membrane protrusion and stress fiber formation. To allow for actin filament growth, more F-actin barbed ends (the sites of actin filament elongation) must become exposed. In platelets, Hartwig *et al* (1995) have demonstrated that Rac does this by stimulating an increase in phoshoinositide phosphorylation. The resulting PIP_2 binds to actin capping proteins and results in the uncapping of actin filaments and the creation of free barbed ends (Hartwig *et al.*,

1995). Additional events influencing actin polymerization and membrane attachment such as altering actin filament capping and cross-linking at adhesion sites, affecting *de novo* nucleation, inducing filament cutting to create free barbed ends, altering the availability of monomeric actin and regulating membrane-cytoskeletal binding sites remain to be linked to these GTPases (Zigmond, 1996). While a few upstream elements and downstream effectors of these GTPases have been identified, the exact pathways involved in cell motility remain to be identified.

The study of cell motility mechanisms by a number of groups has focused on the variety of cytoskeletal building units, critical actin filament capping proteins and their regulation, the production of motile forces, formation and release of adhesions and the signaling involved in all of these events or on particular signaling proteins. In this thesis, there is a focus on the focal adhesion/motility relationship for a number of reasons. First of all, there is a strong correlation between the number, size and strength of focal adhesions within a cell and the amount of cell movement. Generally, stationary cells form strong focal adhesions, whereas more motile cells form fewer or smaller, less organized adhesive structures (Dunlevy & Couchman, 1993). Secondly, many of the signaling molecules within focal adhesions are also involved in the regulation of cell motility (Huttenlocher *et al.*, 1995). Finally, there is a relationship between focal adhesions, cell motility, signaling oncoproteins and tumorigenesis that suggest an intersection of events regulating adhesion, motility and cell growth.

The ECM as a stimulus of growth and differentiation - models: The signals that arise from the interactions at focal adhesions greatly contribute to the regulation of cell shape, gene expression, cellular signaling, cytoskeletal structure, cell motility, growth control and the survival of a cell (reviewed in Damsky & Werb, 1992; Juliano & Haskill, 1993; Ruoslahti & Reed, 1994; Clark & Brugge, 1995; Roskelley *et al.*, 1995). However, because many different pathways intersect at focal adhesions, the cellular state greatly influences signal reception from the ECM. That is, the manner in which a cell receives and responds to these ECM-derived signals depends on a number of different determinants including the cell type, differentiation state, the repertoire of expressed genes, the stage in the cell cycle, the environmental constraints on the cell including cell-cell and other cell-matrix interactions and the presence of other conflicting or cooperating signals from other extracellular factors (see Figure 3). Thus, the ECM can influence the growth and differentiation state of a cell, and the growth and differentiation state of a cell can alter its response to the ECM.

Several laboratories have made the jump from the molecular model of focal adhesion signaling to a whole cell and/or tissue model of how the ECM regulates cell functions. The most prominent of these models are those presented by Mina Bissell's and Donald Ingber's laboratories. These groups have presented interactive models that describe the informational flow between a cell and the ECM. Mina Bissell's group first presented their idea of "dynamic reciprocity" in 1982 (Bissell *et al.*, 1982). In this conceptual model, there is a continuous flow of information from the ECM

to the nucleus and back again (Figure 4; Lin & Bissell, 1993, Roskelley *et al.*, 1995). In a similar model, Donald Ingber has taken an engineering approach when regarding the ECM in his tensigrity model (Figure 5; Ingber, 1993; Wang *et al.*, 1993). In this model, the ECM exerts a mechanical signal that results in a rearrangement of cytoskeletal and nuclear lattices. The modified architecture then influences the molecular machinery of the cell. In both models, a continuum exists between the ECM, the cytoskeleton (including intermediate filaments, actin microfilaments and microtubules[3]) and the nuclear matrix. The binding of cell surface receptors to the ECM stimulates a response that involves assembly of cytoskeletal components at the focal contacts, followed by a coordinated response within the nuclear scaffolds. The structural changes in the ECM-cytoskeleton-nuclear matrix architecture can 1) direct the activation of intracellular signaling cascades and 2) provide a backdrop for the interpretation of additional biochemical signals. In a sense, these models represent the Zen of Cell Biology where a cell is at one with its microenvironment and the ECM cannot be regarded without considering the cytoskeleton, intracellular signaling and gene expression.

[3]Although the focus here is on the actin cytoskeleton, intermediate filaments and microtubules are of equal importance. Intermediate filaments may provide important links between the cytoplasm and the nuclear matrix, while microtubules are of upmost importance for cell division, and are possibly involved in key regulatory pathways.

The Extracellular Matrix in Tumorigenesis, Tumor Cell Locomotion, Invasion and Metastasis

The transformation of normal cells into tumor cells and the progression of tumor cells into metastatic ones involves changes in gene expression, alterations in growth regulation, modification of cell shape, changes in adherence to surrounding cells and to the ECM and the conversion from stationary phase to a migratory and invasive one. Thus it should be apparent that ECM molecules and their receptors are also important regulators of tumor progression and metastasis (Starkey, 1990; Aznavoorian *et al.*, 1993; Giancotti & Maniero, 1994). The ECM can both directly influence events like gene expression, growth and motility and indirectly can regulate cellular responses to growth factors and cytokines (Figure 3). As a result, the ECM and their receptors are promising to be important clinical targets to block tumor growth and invasion (Kohn & Liotta, 1995). In this section I will give an introduction to the role of the ECM in transformation and metastasis.

Characteristics of normal and metastatic cells: When normal cells are grown in culture they have a finite life span; they exhibit contact-inhibition[4], and hence a low nuclear overlap ratio; they usually have a high growth requirement for serum; and they exhibit anchorage-dependent cell growth (Folkman & Moscona, 1978; Grinnel, 1978; Abercrombie, 1980; Stoker *et al.*, 1983). In contrast, tumor cells grown in

[4]Contact inhibition is defined as the cessation of movement and cell division when a confluent monolayer is formed.

culture have an indefinite life span; do not exhibit contact inhibition, and hence continue to move and grow after a monolayer is formed; exhibit higher nuclear overlap ratios; can exhibit anchorage independent growth; and can grow in the absence of serum growth factors. The transformation of normal cells into neoplastic ones involves numerous changes over time, and *in vivo* transformation is referred to as tumor progression. ECM molecules, their receptors and ECM degradation enzymes are important to the process of tumor progression. In some cases, ECM molecules and their receptors can act as oncogenes by regulating or directing transformation/tumorigenesis (e.g. RHAMM, Chapter 2).

Adhesion, growth, cell motility and transformation: Cellular adhesion, cell growth and migration are highly interconnected, but distinct, processes (Giancotti & Mainiero, 1994). It has been noted that when cells strongly adhere to the surrounding ECM, growth and migration (and/or invasion) are largely restricted. Contrarily, cells that do not adhere to a matrix or to other cells cannot migrate over a substratum, nor does growth normally occur. When there is moderate adhesion to the ECM, with the ability to detach and reattach, cells can locomote and grow (see Figure 3; Giancotti & Mainiero, 1994). Thus neoplastic cells tend to belong to the moderately adhesive group, or they escape the confines of normal cell growth. Only when there is a balance of adhesion/actin cytoskeleton assembly/disassembly and adhesion/de-adhesion does cell locomotion (and/or cell invasion) and growth transpire (Figure 3).

During transformation, or after the addition of a mitogen or motogen, there is an exchange of well developed focal adhesions for smaller or more dynamic

adhesion structures often referred to as podosomes. In culture, podosomes look like small punctate focal adhesions. *In vivo*, podosomes are three dimensional structures that not only represent sites of adhesion but sites of proteolytic enzyme secretion and cell invasion. These small focal adhesions of transformed cells are associated with growth and motility and can be promoted by factors like platelet-derived growth factor, PDGF, (Ridley & Hall, 1992), ECM receptors like RHAMM (Hall *et al.*, 1994), or ECM molecules like thrombospondin and tenascin (Murphy-Ullrich & Hook, 1989; Murphy-Ullrich *et al.*, 1991). Formation of larger focal adhesions can be induced by some integrin:ECM interactions, addition of the neuropeptides (e.g. LPA and bombesin), or addition of growth factors (e.g. epidermal growth factor, EGF). As well, the genetic manipulation of the cytoskeleton can affect both focal adhesion formation and size and can affect transformation. Focal adhesion assembly promoted by the overexpression of tensin, vinculin and α-actinin causes partial reversion of transformation and decreased cell motility. A decrease in protein expression or interference with of α-actinin or vinculin results in fewer focal adhesions (more podosome-like structures) and promotes cell locomotion and transformation dependent properties (Gluck *et al.*, 1993; Rodriguez Fernandez *et al.*, 1993; Gluck & Ben-Ze'ev, 1994; Lo & Chen, 1994). In most cases, actin assembly and focal adhesion formation is associated with arrested movement and growth, while actin disassembly and de-adhesion is associated with the ability of transformed cells, but not normal cells, to grow.

ECM adhesion in metastasis and invasion: Metastasis is a multi-step process that involves adhesive interactions and cell locomotion. The first stage is the escape of tumor cells from the primary tumor. This stage includes a decrease in adhesiveness to the surrounding tumor cells, the release of matrix degrading enzymes and the adherence and migration of tumor cells through the degraded matrix (Stetler-Stevenson *et al.*, 1993). The next stages include the entrance of invading cells into the blood stream, the arrest and extravasation of these cells at target organs and the proliferation of tumor cells at the metastasis site. Since ECM:tumor cell interactions play an important role in all of these steps, the interference or enhancement of adhesion can hinder or induce the invasion of tumor cells. For example, the addition of peptides that mimic ECM molecules, can promote adhesion or interfere with ECM:receptor binding and can also inhibit tumor invasion (Barsky *et al.*, 1984; Humphries *et al.*, 1986, 1988; Gehlsen *et al.*, 1988; Saiki *et al.*, 1989). Changes in ECM receptor levels also contribute to invasion and metastasis: the expression of certain variant isoforms of CD44 can bestow a metastatic phenotype on previously benign, tumorigenic cells (Gunthert *et al.*, 1991; Rudy *et al.*, 1993); RHAMM expression has a positive effect on metastasis (see Chapter 2); and enhanced expression of the $\alpha_v\beta_3$ vitronectin receptor is associated with tumorigenesis and metastasis (Albelda *et al.*, 1990; Gehlsen *et al.*, 1992). These receptors can elicit their effects directly by influencing cell adhesion during invasion, or indirectly by signaling other events. Indeed the $\alpha_v\beta_3$ receptor can signal the protease expression that enhances invasion (Seftor et al., 1992). Enhanced matrix degradation by

proteases and heparinases not only allows space for cells to invade, but the matrix breakdown products and released local factors may act as chemokines or motogens, promoting further adhesion and invasion (Stetler-Stevenson *et al.*, 1993).

Cell motility in invasion and metastasis: The relationship between metastasis and cell locomotion is an intuitive one. Tumor cells that are known to be invasive and metastatic have a higher rate of motility than do their non-metastatic counterparts. This motility parameter can be measured as random cell locomotion, chemotaxis in Boyden chambers, haptotaxis along matrix gradients, the degree of pseudopod extension or the amount of membrane ruffling (Aznavoorian *et al.*, 1993). Because of the connection between cell motility rates and metastasis, agents that promote cell locomotion are logical targets to block tumor metastasis, a leading cause of tumor death. However the variety and diversity of agents that stimulate tumor cell locomotion, such as host-derived scatter factors (e.g. scatter factor or hepatocyte-derived growth factor), growth factors (e.g. NGF, FGF, IGF), ECM components (e.g. laminin, fibronectin, thrombospondin, hyaluronan), tumor secreted factors (e.g. autocrine motility factor) and intracellular oncogenes (e.g. *ras*) [Starkey, 1990; Aznavoorian *et al.*, 1993] make targeting cell motility difficult. Furthermore, while motility is necessary for tumor invasion, stimulation of cell motility is not sufficient to trigger tumor progression and invasion. As a result, the intracellular pathways stimulated by motility factors, mitogens and oncogenes have been examined for common components. Blocking pathways that are necessary for both cell motility and cell growth, for example, may be the ideal strategy for blocking malignancy.

Using this approach, this thesis addresses the intracellular pathways involved in HA:RHAMM mediated cell motility in tumor cells.

Figures

Figure 1. RHAMM Gene Structure and Protein Isoforms: A. The murine RHAMM gene occurs as a single copy gene spanning at least 20 kb on mouse chromosome 11. The gene is comprised of 14 exons ranging in size from 75 to 1099 bp and 13 introns ranging from 90 to 6050 bp. The hatched regions represent untranslated regions, R designates exon 8 encoding the repeat sequence, and HA1 and HA2 mark exons 12 and 13 designating the two HA binding regions. **B.** The three murine RHAMM isoforms identified to date are RHAMM1, RHAMM1v4 and RHAMM2. All identified exons are encoded in RHAMM1v4, while RHAMM1 is missing exon 4 and RHAMM2 is truncated, encoded by exons 6-14. RHAMM1 and RHAMM1v4 contain a potential signal sequence (SS) at the amino terminus of the protein. All isoforms contain the repeat sequence (R) containing 5 of the 9 potential N-glycosylation sites, 2 HA binding domains (HA) and a carboxy terminus hydrophillic amino acid sequence (GPI) as a potential lipid attachment site. The RHAMM proteins are predominantly hydrophillic and are predicted to occur as α-helices (Entwistle *et al.*, 1995).

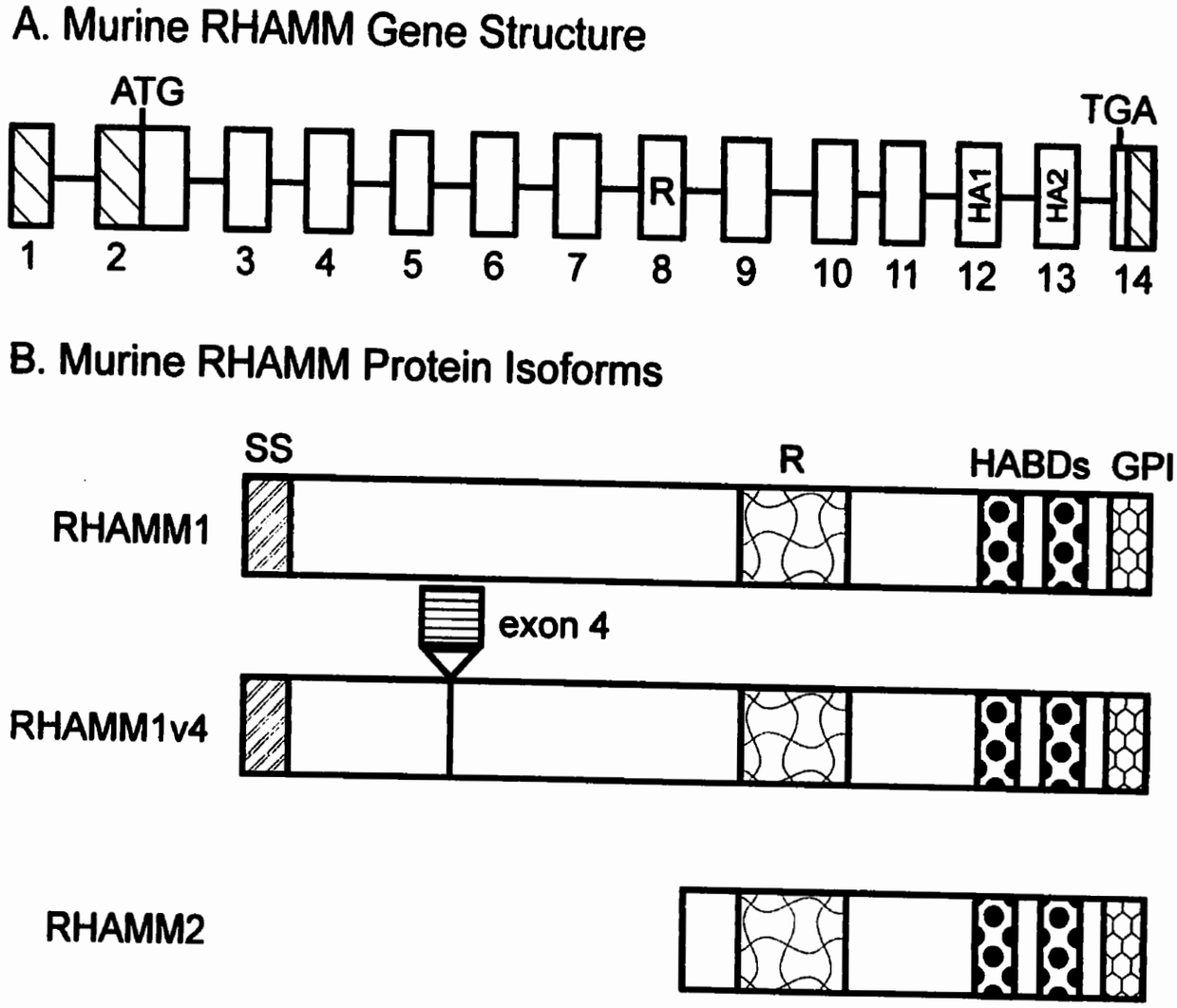

A. Murine RHAMM Gene Structure
ATG
TGA
R
HA1
HA2
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B. Murine RHAMM Protein Isoforms
SS
R
HABDs
GPI
RHAMM1
exon 4
RHAMM1v4
RHAMM2

Figure 1.

Figure 2. Focal Adhesion Structure: A model of the protein-protein interactions at focal adhesions. Integrins adhere extracellularly to the ECM and intracellularly to the cytoskeleton. Cytoskeletal proteins assemble in a precise manner to form focal adhesion structures. Signaling proteins are recruited to focal adhesions and influence assembly and disassembly. See the text for a detailed description. Reproduced from Luna & Hitt. (1992). *Science* **258**, page 960.

Figure 2

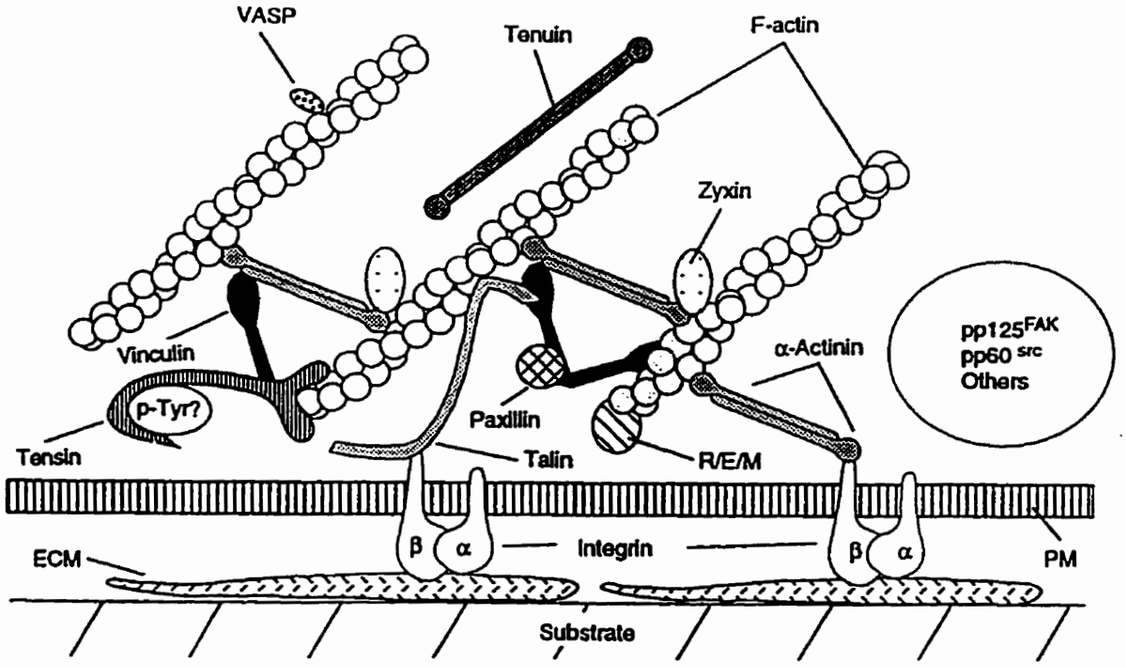


Fig. 3. Working model of the protein-protein interactions in focal adhesions determined by in vitro–binding experiments and immunolocalization. Most associations have not yet been verified by in vivo studies. In addition, several interactions are of relatively low affinity in solution, but may be enhanced due to the reduction in dimensionality at the membrane surface (*100*). Abbreviations are: ECM, extracellular matrix; PM, plasma membrane; p-Tyr-?, unknown phosphotyrosine-containing protein; R/E/M, member of the radixin/ezrin/moesin family; VASP, vasodilator-stimulated phosphoprotein. Diagram is modified from Simon *et al.* (*58*) with permission.

Figure 3. The Cellular Circuit Board: A number of different cellular inputs affect the signal transduction pathways that intersect at focal adhesions. The response of the cell greatly depends upon its current cell-cell interactions, the constraints of cellular shape, the panel of ECM receptors expressed and its surrounding matrix composition, the presence of growth factors, hormones and neuropeptides, the current expression of regulatory molecules and intracellular oncogenes, and the stage of cell cycle progression. All of these inputs can influence the output from any one signal. For example, increased or decreased adhesion due to the presence of a growth factor or an ECM component can influence the amount of actin assembly/disassembly which in turn can influence cell locomotion and cell growth. For cell movement, a balance of assembly and disassembly must be accompanied by the expression of appropriate regulatory molecules, ECM receptors and cytoskeleton proteins, and coordinated with adhesion/de adhesion to the surrounding cells and matrix. Alternatively, if the balance of contributing signals culminates in cell cycle progression and cell division, changes in adhesion, cytoskeletal assembly (actin, intermediate filaments and microtubules) and cell shape results. Conversely, cell cycle arrest and quiescence is associated with a taut cytoskeletal network and defined cell-cell and cell-matrix adhesions.

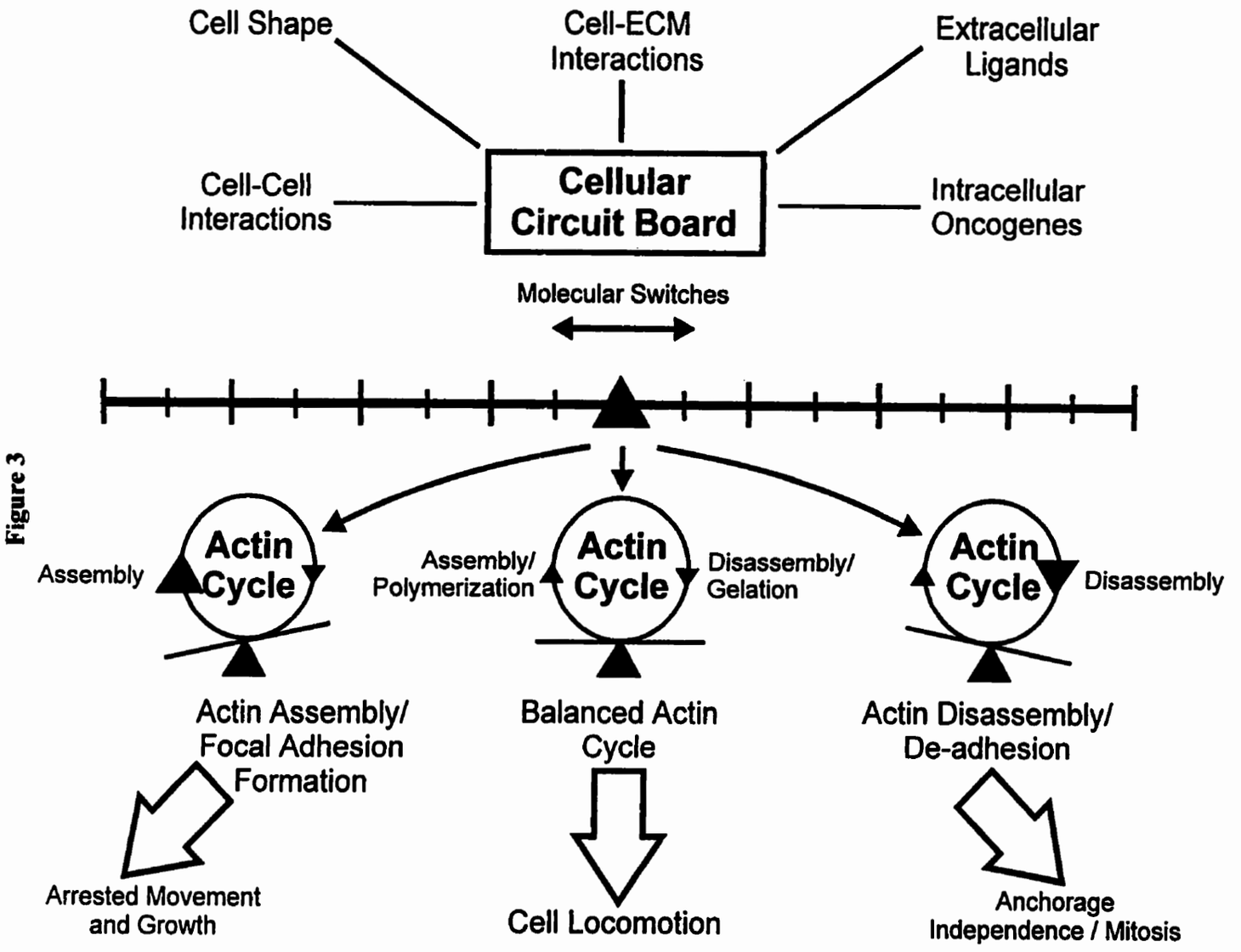

Cell Shape
Cell-ECM Interactions
Extracellular Ligands
Cell-Cell Interactions
Cellular Circuit Board
Intracellular Oncogenes
Molecular Switches
Assembly
Actin Cycle
Assembly/ Polymerization
Actin Cycle
Disassembly/ Gelation
Actin Cycle
Disassembly
Actin Assembly/ Focal Adhesion Formation
Balanced Actin Cycle
Actin Disassembly/ De-adhesion
Arrested Movement and Growth
Cell Locomotion
Anchorage Independence / Mitosis

Figure 3

Figure 4. Mina Bissell's Dynamic Reciprocity Model: A model of ECM/cytoskeleton input into gene expression and cell differentiation. Reproduced from Lin & Bissell, 1993. *FASEB J.* **7**, page 738.

Figure 4

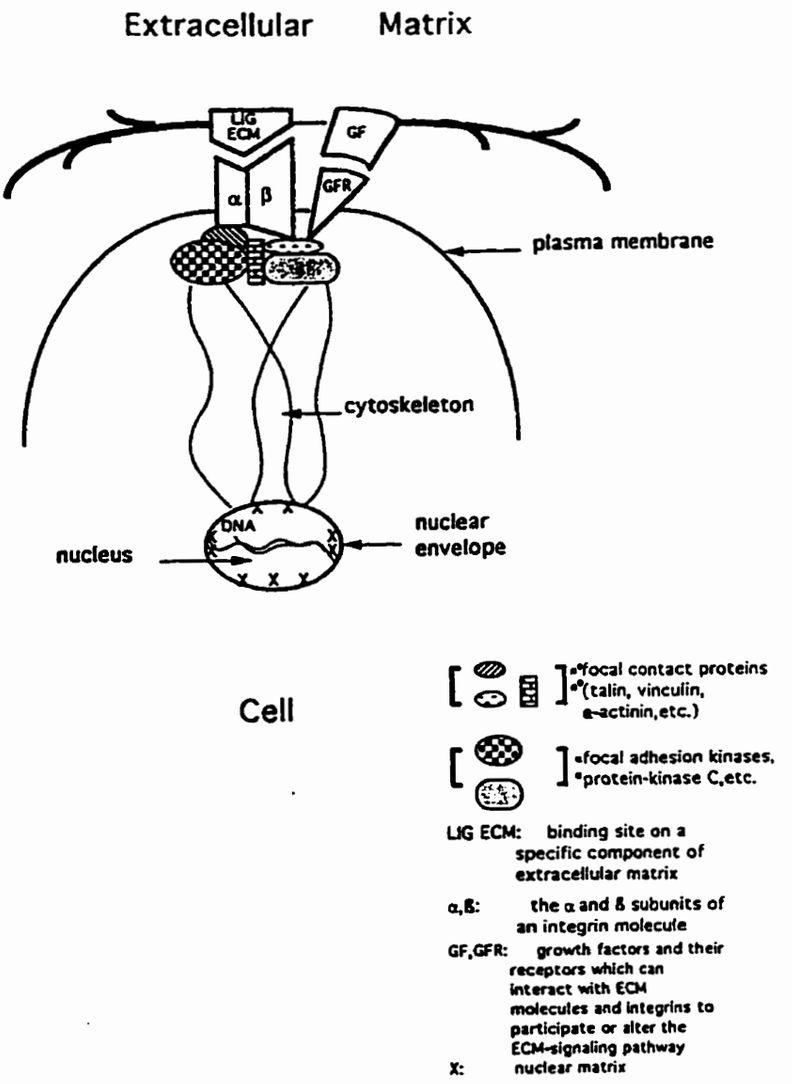


Figure 1. General hypothesis for the mechanism by which extracellular matrix regulates gene expression and differentiation. Extracellular molecules bind to their specific receptors (here shown as an integrin), triggering changes in the cytoplasmic domain of the receptor, which in turn causes appropriate assembly of the focal contact proteins and multiple phosphorylation of other intracellular components. These changes can bring about rearrangement of the cytoskeleton, which its their connection with the nucleus can trigger differential interaction of the chromatin with the nuclear matrix or differential activation of transcription factors (modified from refs 1 and 2).

Figure 5. Donald Ingber's Tensegrity Model: A mechanical model of the effect of ECM adhesion and cytoskeletal architecture on cellular function. Reproduced from Ingber, 1993. *Cell* **75**, page 1250.

Figure 5

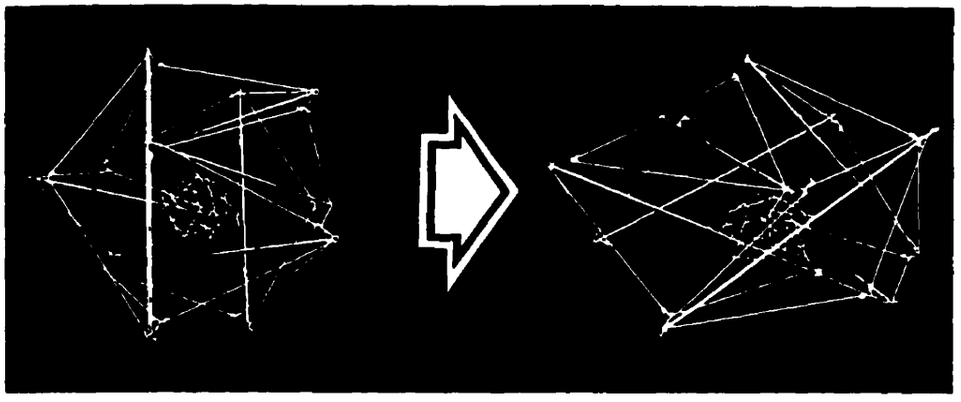


Figure 1. Tensegrity Cell Model

Mechanical stresses associated with the formation of basal cell–substratum adhesions cause structural elements throughout the depth of a nucleated tensegrity cell model to rearrange in a coordinated manner. Similar changes occur in living cells when they attach to extracellular matrix. Use of tensegrity architecture by cells could provide a mechanism to distribute mechanical stresses to regulatory molecules and metabolic enzymes that are immobilized on cytoskeletal and nuclear scaffolds and thereby to integrate cell structure and function (for further discussion see Ingber, 1993; Wang et al., 1993).

Cell, Vol. 75, 1249–1252, December 31, 1993, Copyright © 1993 by Cell Press

References

Abercrombie M. (1980). The crawling movement of metazoan cells. *Proc. R. Soc. Lond. (Biol)*, **207**, 109-147.

Albelda SM, Matte SA, Elder DE, Stewart R, Damjanovich L, Herlyn M. (1990). Integrin distribution in malignant melanoma: Association of the β3 subunit with tumor progress. *Cancer Res.*, **50**, 6757-6764.

Anderson D, Koch CA, Grey L, Ellis C, Moran MF and Pawson T. (1990). Binding of SH2 domains of phospholipase C gamma1, GAP and Src to activated growth factor receptors. *Science*, **250**, 979-982.

Anderson RWG. (1994). Functional specialization of the glycosylphosphatidyl-inositol membrane anchor. *Semin. Immunol.*, **6**, 89-95.

Aruffo A, Stamenkovic I, Menick M, Underhill CB and Seed B. (1990). CD44 is the principal cell surface receptor for hyaluronate. *Cell*, **61**, 1303-1313.

Aznavoorian S, Murphy AN, Stetler-Stevenson WG and Liotta LA. (1993). Molecular aspects of tumor cell invasion and metastasis. *Cancer*, **71**, 1368-1383.

Barsky SH, Rao CN, Williams JE and Liotta LA. (1984). Laminin molecular domains which alter metastasis in a murine model. *J. Clin. Invest.*, **74**, 843-848.

Bergman M, Joukov V, Virtanen I and Alitalo K. (1995). Overexpressed Csk tyrosine kinase is localized in focal adhesions, causes reorganization of $\alpha_v\beta_5$ integrin, and interferes with HeLa cell spreading. *Mol Cell. Biol.*, **15**, 711-

722.

Bernfield M, Kokenyasi R, Kato M, Hinkes MT, Spring J, Gallo R and Lose EJ. (1992). Biology of heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.*, **8**, 365-393.

Birge R, Fajardo JE, Reichman C, Shoelson SE, Songyang Z, Cantley LC and Hanafusa H. (1993). Identification and characterization of a high affinity interaction between v-crk and tyrosine-phosphorylated paxillin in CT10-transformed fibroblasts. *Mol. Cell. Biol.*, **13**, 4648-4656.

Bishop JM. (1991). Molecular themes in oncogenesis. *Cell*, **64**, 235-248.

Bissell MJ, Hall HG and Parry G. (1982). How does extracellular matrix direct gene expression? *J. Theor. Biol.*, **99**, 31-68.

Boudreau N, Turley EA and Rabinovitch M. (1991). Fibronectin, hyaluronan and a hyaluronan bindingprotein contribute to increased ductus arteriosis smooth muscle cell migration. *Dev. Biol.*, **143**, 235-247.

Bourguignon LYW, Kalomiris E and Lokeshwar VB. (1991). Post-translational modification and expression of ankyrin-binding site(s) in GP85 (Pgp-1, CD44) and its biosynthesis precursors during T-lymphoma membrane biosynthesis. *J. Biol. Chem.*, **266**, 17983-17989.

Buck CA and Horwitz AF. (1987). Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.*, **3**, 179-205.

Burridge K, Fath K, Kelly T, Nuckolls G and Turner C. (1988). Focal adhesions: Transmembrane junctions between the extracellular matrix and the

cytoskeleton. *Annu. Rev. Cell Biol.*, **4**, 487-525.

Burridge K, Turner CE and Romer LH. (1992). Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.*, **119**, 893-903.

Cantley LC, Auger K, Carpenter C, Duckworth B, Graziani A, Kapeller R and Soltoff S. (1991). Oncogenes and signal transduction. *Cell*, **64**, 281-302.

Cantley LC and Songyang Z. (1994). Specificity in recognition of phosphopeptides by src-homology 2 domains. *J. Cell Sci.*, **Suppl 18**, 121-126.

Carter WG and Wayner EA. (1988). Characterization of the class III collagen receptor, a phosphorylated, transmembrane glycoprotein expressed in nucleated cells. *J. Biol. Chem.*, **263**, 4193-4201.

Chan BMC, Matsuura M, Takada Y, Zetter BR and Hemler ME. (1991). *In vitro* and *in vivo* consequences of VLA-2 expression on rhabdosarcoma cells. *Science*, **251**, 1600-1602.

Chen Q, Kinch MS, Lin TH, Burridge K and Juliano RL. (1994). Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J. Biol. Chem.*, **269**, 26602-26606.

Chen Q, Lin TH, Der CJ and Juliano RL. (1996). Integrin-mediated activation of mitogen-activated protein (MAP) or extracellular signal-related kinase kinases (MEK) and kinase is independent of ras. *J. Biol. Chem.*, **271**, 18122-18127.

Clark EA and Brugge JS. (1995). Integrins and signal transduction pathways: the

road taken. *Science*, **268**, 233-239.

Cobb BS, Schaller MD, Leu T-H and Parsons JT. (1994). Stable association of $pp60^{src}$ and $p59^{fyn}$ with the focal adhesion-associated protein tyrosine kinase, $pp125^{FAK}$. *Mol. Cell. Biol.*, **14**, 147-155.

Cohen GB, Ren R and Baltimore D. (1995). Modular binding domains in signal transduction proteins. *Cell*, **80**, 237-248.

Craig SW and Johnson RP. (1996). Assembly of focal adhesions: progress, paradigms, and portents. *Curr. Opin. Cell Biol.*, **8**, 74-85.

Culty M, Nguyen HA and Underhill CB. (1992). The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan. *J. Cell Biol*, **116**, 1055-1062.

Damsky C and Werb Z. (1992). Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr. Opin. Cell Biol.*, **4**, 772-781.

David G. (1993). Integral membrane heparan sufate proteoglycans. *FASEB J.*, **7**, 1023-1030.

Dunlevy JR and Couchman JR. (1993). Controlled induction of focal adhesion disassembly and migration in primary fibroblasts. *J. Cell Sci.*, **105**, 489-500.

Entwistle J, Hall CL and Turley EA. (1996). HA receptors: regulators of signalling to the cytoskeleton. *J. Cell. Biochem.*, **61**,569-577.

Entwistle J, Zhang S, Yang B, Wong C, Li Q, Hall CL, A J, Mowat M, Greenberg AH and Turley EA. (1995). Characterization of the murine gene encoding

the hyaluronan receptor RHAMM. *Gene*, **163**, 233-238.

Felding-Habermann B, Mueller BM, Romerdahl CA and Cheresh DA. (1992). Involvement of integrin alpha V gene expression in human melanoma tumorigenicity. *J. Clin. Invest.*, **89**, 2018-2022.

Folkman J and Moscona A. (1978). Role of cell shape in growth control. *Nature*, **273**, 345-349.

Gehlsen KR, Argraves WS, Pierschbacher MD and Ruoslahti E. (1988). Inhibition of *in vitro* tumor cell invasion by Arg-Gly-Asp containing synthetic peptides. *J. Cell Biol.*, **106**, 925-930.

Gehlsen KR, Davis GE and Sriramarao P. (1992). Integrin expression in human melanoma cells with differing invasive and metastatic properites. *Clin. Exp. Metastasis*, **10**, 111-120.

Giancotti FG and Mainiero F. (1994). Integrin-mediated adhesion and signaling in tumorigenesis. *Biochim. Biophys Acta*, **1198**, 47-64.

Giancotti FG and Ruoslahti E. (1990). Elevated levels of the α5β1 fibronectin receptor suppress the transformed phenotype of CHO cells. *Cell*, **60**, 849-859.

Gluck U and Ben-Ze'ev A. (1994). Modulation of α-actinin levels affects cell motility and confers tumorigenicity on 3T3 cells. *J. Cell Sci.*, **107**, 1773-1782.

Gluck U, Kwiatkowski DJ and Ben-Ze'ev A. (1993). Suppression of tumorigenicity in simian virus 40-transformed 3T3 cells transfected with α-actinin cDNA. *Proc. Natl. Acad. Sci.*, **90**, 383-387.

Grinnel F. (1978). Cellular adhesiveness and extracellular substrate. *Int. Rev. Cytol.*, **53**, 65-74

Guan J-L, Trevithick JE and Hynes RO. (1991). Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120 kD protein. *Cell Regul.*, **2**, 951-964.

Gumbiner BM. (1996) Cell adhesion: The molecular basis of tissue architecture and morphogenesis. *Cell*, **84**, 345-357.

Gunthert U. (1993). CD44: multitude of isoforms with diverse functions. *Curr. Topics Microbiol. Immunol.*, **184**, 47-63.

Gunthert U, Hoffman M, Rudy W, Reber S, Zoller M, Haubmann I, Matzhu S, Wenzel A, Ponta H and Herrlich P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell*, **65**, 13-24.

Hall CL and Turley EA. (1995). Hyaluronan: RHAMM mediated cell locomotion and signaling in tumorigenesis. *J. Neuro-Oncol.*, **26**, 221-229.

Hall CL, Wang C, Lange LA and Turley EA. (1994). Hyaluronan and the hyaluronan receptor RHAMM protmote focal adhesion turnover and transient tyrosine kinase activity. *J. Cell Biol.*, **126**, 575-588.

Hall CL, Yang B, Yang X, Zhang S, Turley M, Samuel S, Lange LA, Wang C, Curpen GD, Savani RC, Greenberg AH and Turley EA. (1995). Overexpression of the hyaluronan receptor RHAMM is transforming and is also required for H-*ras* transformation. *Cell*, **82**, 19-28.

Hanks SK, Calalb MB, Harper MC and Patel SK. (1992). Focal adhesion protein

tyrosine kinase phosphorylated in response to cell spreading on fibronectin. *Proc. Natl. Acad. Sci.*, **89**, 8487-8491.

Hansen LK, Mooney DJ, Vacanti JP and Inger DE. (1994). Integrin binding and cell spreading on extracellular matrix act at different points in the cell cycle to promote hepatocyte growth. *Mol. Biol. Cell*, **5**, 967-975.

Hardwick C, Hoare K, Owens R, Hohn HP, Hook M, Moore D, Cripps V, Austen L, Nance DM and Turley EA. (1992). Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. *J. Cell. Biol.*, **117**, 1343-1350.

Hart IR, Birch M and Marshall JF. (1991). Cell adhesion receptor expression during melanoma progression and metastasis. *Cancer Metastasis Rev.*, **10**, 115-128.

Hartwig JH, Bokoch GM, Carpenter CL, Janmey PA, Taylor LA, Toker A and Stossel TP. (1995). Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell*, **82**, 643-653.

Haynes BF, Telen MJ, Hale LP and Denning SM. (1989). CD44- a molecule involved in leukocyte adherence and T-cell activation. *Immunol. Today*, **10**, 423-428.

He Q, Lesley J, Hyman R, Ishihara K and Kincade PW. (1992). Molecular isoforms of murine CD44 and evidence that the membrane proximal domain is not critical for hyaluronate recognition. *J. Cell. Biol.*, **119**, 1711-1719.

Hua Q, Knudson CB and Knudson W. (1993). Internalization of hyaluronan by chon-

drocytes occurs via receptor-mediated endocytosis. *J. Cell Sci.*, **106**, 365-375.

Humphries MJ, Olden K and Yamada KM. (1986). A synthetic petide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science*, **233**, 467.

Humphries MJ, Yamada KM and Olden K. (1988). Investigation of the biological effects of anti-cell adhesive synthetic peptides that inhibit experimental metastasis of B16 F10 murine melanoma cells. *J. Clin. Invest.*, **81**, 782-790.

Huttenlocher A, Sanborg RR and Horwitz AF. (1995). Adhesion in cell migration. *Curr. Opin. Cell Biol.*, **7**, 697-706.

Hynes RO. (1992). Integrins: versatility, modulation and signaling in cell adhesion. *Cell*, **69**, 11-25.

Hynes RO. (1994). The impact of molecular biology on models for cell adhesion. *BioEssays*, **16**, 663-669.

Ingber DE. (1993). The riddle of morphogenesis: a question of solution chemistry or molecular cell engineering? *Cell*, **75**, 1249-1252.

Jaken , SK, Leach K and Klauck T. (1989). Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. *J. Cell Biol.*, **109**, 697-704.

Jalkanen S, Bargatze RF, de los Toyos J and Butcher EC. (1987). Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-90 kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosyl, or synovial endothelial cells. *J. Cell Biol.*, **105**, 983-990.

Jalkanen S and Jalkanen M. (1992). Lymphocyte CD44 binds the COOH-terminal

heparin-binding domain of fibronectin. *J. Cell Biol.*, **116**, 817-825.

Jamney PA. (1994). Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu. Rev. Physiol.*, **56**, 169-191.

Jockusch BM, Bubeck P, Giehl K, Kroemker M, Moscher J, Rothkegal M, Rudiger M, Scluter K, Stanke G and Winkler J. (1995). The molecular architecture of focal adhesions. *Annu. Rev. Cell Dev. Biol.*, **11**, 379-416.

Juliano RL and Haskill S. (1993). Signal transduction from the extracellular matrix. *J. Cell Biol.*, **120**, 577-585.

Kalomiris EL and Bourguignon LYW. (1989). Lymphoma protein kinase C is associated with the transmembrane glycoprotein, GP85 and may function in GP85-ankyrin binding. *J Biol. Chem.*, **264**, 8113-8119.

Kapron-Bras C, Fitz-Gibbon L, Jeevaratnam P, Wilkins J and Dedhar S. (1993). Stimulation of tyrosine phosphorylation and accumulation of GTP-bound p21ras upon antibody-mediated $\alpha_2\beta_1$ integrin activation in T-lymphoblastic cells. *J Biol. Chem.*, **268**, 20701-20704.

Klewes L, Turley EA and Prehm P. (1993). The hyaluronate sunthase from a eukaryotic cell line. *Biochem J.*, **290**, 791-795.

Knudson W, Biswas C, Li X-Q, Nemec RE and Toole BP. (1989). The role and regulation of tumour associated hyaluronan. *Ciba Found. Symp: Biology of Hyaluronan*, **143**, 150-169.

Knudson CB and Knudson W. (1993). Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *FASEB J.*, **7**, 1233-1241.

Kohn EC and Liotta LA. (1995). Molecular insights into cancer invasion: stratagies for prevention and intervention. *Cancer Res.*, **55**, 1856-1862.

Kornberg LJ, Earp HS, Turner CE, Prockop C and Juliano RL. (1991). Signal transduction by integrins: Increased protein tyorsine phosphorylation caused by integrin clustering. *Proc. Natl. Acad. Sci.*, **88**, 8392-8396.

Kornovski BS, McCoshen J, Kredentser J and Turley EA. (1994). The regulation of sperm motility by a novel hyaluronan receptr. *Fertil. Steril.*, **61**, 935-940.

Lacy BE and Underhill CB. (1987). The hyaluronate receptor is associated with actin filaments. *J. Cell Biol.*, **105**, 1395-1404.

Laurant TC and Fraser JRE. (1992). Hyaluronan. *FASEB J.*, **6**, 2397-2404.

Lee TH, Klampfer L, Shows TB and Vileck J. (1993). Transcriptional regulation of TSG-6, a tumor necrosis factor- and interleukin-1-inducible primary response gene coding for a secreted hyaluronan binding protein. *J. Biol. Chem.*, **268**, 6154-6160.

Lee TH, Wisniewski HG and Vilcek J. (1992). A novel secretory tumor necrosis factor-inducible protein (TSG-6) is a member of the family of hyaluronate binding proteins, closely related to the adhesion receptor CD44. *J. Cell Biol.*, **116**, 545-557.

Lesley J, Howes N, Perschl A and Hyman R. (1994). Hyaluronan binding function of CD44 is transiently activated on T cells during an in vivo immune response. *J. Exp. Med.*, **180**, 383-387.

Lesley J and Hyman R. (1992). CD44 can be activated to function as an hyaluronic

acid receptor in normal murine T cells. *Eur. J. Immunol.*, **22**, 2719-2723.

Liao HX, Levesque MC, Patton K, Bergamo B, Jones D, Moody MA, Telen MJ and Haynes BF. (1993). Regulation of human CD44H and CD44E isoform binding to hyaluronan by phorbol myristate acetate and anti-CD44 monoclonal and polyclonal antibodies. *J. Immunol.*, **151**, 6490-6499.

Lin CQ and Bissell MJ. (1993). Multifaceted regulation of cell differentiation by extracellular matrix. *FASEB J.*, **7**, 737-743.

Lo SH and Chen LB. (1994). Focal adhesion as a signal transduction organelle. *Cancer Metastasis Rev.*, **13**, 9-24.

Lokeshwar VB and Bourguignon LYW. (1992). The lymphoma transmembrane glycoprotein GP85 (CD44) is novel guanine nucleotide-binding protein which regulates GP85 (CD44)-ankyrin interaction. *J. Biol. Chem.*, **267**, 22073-22078.

Lokeshwar VB, Fregien N and Bourguignon LYW. (1994). Ankyrin-binding domain of CD44-(GP85) is required for the expression of hyaluronic acid mediated adhesion function. *J. Cell. Biol.*, **126**, 1099-1109.

Luna EJ and Hitt AL. (1992). Cytoskeletal-plasma membrane interactions. *Science*, **258**, 955-964.

Marengere LEM and Pawson T. (1994). Structure and function of SH2 domains. *J. Cell Sci.*, **Suppl 18**, 97-104

Masellis-Smith A, Belch AR, Mant MJ, Turley EA and Pilarski LM. (1996). Hyaluronan-dependent motility of B cells and leukemic plasma cells in blood,

but not of bone marrow plasma cells, in multiple myeloma: Alternate use of receptor for hyaluronan-mediated motility (RHAMM) and CD44. *Blood*, **87**, 1891-1899.

Marshall MS. (1995). Ras target proteins in eukaryotic cells. *FASEB J.*, **9**, 1311-1318.

Matsuda M, Mayer B, Fukui Y and Hanafusa H. (1990). Binding of transforming protein p47gag-crk to a broad range of phosphotyrosine-containing proteins. *Science*, **248**, 1537-1539.

McCarthy JB, Skubitz AP, Palm SL and Furcht LT. (1988). Metastasis inhibition of different tumor types by purified fragments of laminin and heparin binding fragment of fibronectin. *J. Natl. Cancer Inst.*, **80**, 108-116.

Mitchison TJ and Cramer LP. (1996). Actin-based cell motility and cell locomotion. *Cell*, **84**, 371-379.

Moran, MF, Koch CA, Anderson D, Ellis C, England L, Martin GS and Pawson T. (1990). Src homology region 2 domains direct protein-protein interactions in signal transduction. *Proc. Natl Acad. Sci.*, **87**, 8622-8626.

Murphy-Ullrich JE and Hook M. (1989). Thrombospondin modulates focal adhesions in endothelial cells. *J. Cell Biol.*, **109**, 1309-1312.

Murphy-Ullrich JE, Lightner VA, Aukhil I, Yan YZ, Erickson HP and Hook M. (1991). Focal adhesion integrity is downregulated by the alternatively spliced domain of human tenascin. *J. Cell Biol.*, **115**, 1127-1136.

Nagy JI, Hacking J, Frankenstein UN and Turley EA. (1995). Requirement of the

hyaluronan receptor RHAMM in neurite extension and motility as demonstrated in primary neurons and neuronal cell lines. *J. Neurosci.*, **15**, 241-252.

Naujokas MF, Morin M, Anderson MS, Peterson M and Miller J. (1993). The chondroitin sulfate form of invariant chain can enhance stimulation of T cell response through interaction with CD44. *Cell*, **74**, 257-268.

Neer EJ. (1995). Heterotrimeric G proteins: Organizers of transmembrane signals. *Cell*, **80**, 249-257.

Nelson RM, Venot A, Bevilacqua MP, Linhardt RJ and Stamenokovic I. (1995). Carbohydrate-protein interactions in vascular biology. *Annu. Rev. Cell Dev. Biol.*, **11**, 601-631.

Nobes CD and Hall A. (1995). Rho, rac and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*, **81**, 53-62.

Nurse P. (1990). Universal control mechanism regulating onset of M-phase. *Nature*, **344**, 503-508.

Opus M. (1995). Cellular adhesiveness, contractility, and traction: stick, grip and slip control. *Biochem. Cell Biol.*, **73**: 311-316.

Parsons JT, Schaller MD, Hildebrand J, Leu T-H, Richardson A and Otey C. (1994). Focal adhesion kinase: structure and signalling. *J. Cell Sci.*, **Suppl. 18**, 109-113.

Peach RJ, Hollenbaugh D, Stamenkovic I and Aruffo A. (1993). Identification of

hyaluronic acid binding sites in the extracellular domain of CD44. *J. Cell Biol.*, **122**, 257-264.

Picker LJ, Nakache M and Butcher EC. (1989). Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules in diverse cell types. *J. Cell Biol.*, **109**, 927-937.

Pilarski LM, Masellis-Smith A, Belch AR, Yang B, Savani RC and Turley EA. (1994). RHAMM, a receptor for hyaluronan mediated motility, on normal human lymphocytes, thymocytes and malignant B cells: a mediator in B cell malignancy? *Leuk. Lymphoma*, **14**, 363-374.

Pilarski LM, Mista H and Turley EA. (1993). Regulated expression of a receptor for hyaluronan-mediated motility on human thymocytes and T cells. *J. Immunol.*, **150**, 4292-4302.

Ploug M, Ronne E, Behrendt N, Jensen AL, Blasi F and Dano K. (1991). Cellular receptor for urokinase plasminogen activator. Carboxyl-terminal processing and membrane anchoring by glycosyl-phosphatidyl-inositol. *J. Biol. Chem.*, **266**, 1926-1933.

Qian F, Vaux DL and Weissman FL. (1994). Expression of the integrin $\alpha 4\beta 1$ on melanoma cells can inhibit the invasive stage of metastasis formation. *Cell*, **77**, 335-347.

Reber S, Matzhu S, Gunthert U, Ponta H, Herrlich P and Zoller M. (1990). Retardation of metatstatic tumor growth after immunization with metastasis-specific monoclonal antibodies. *Int. J. Cancer*, **46**, 919-927.

Resh MD. (1993). Interaction of tyrosine kinase oncoproteins with cellular membranes. *Biochim. Biophys. Acta,* **1155**, 307-322.

Richardson A and Parsons JT. (1995). Signal transduction through integrins: a central role for focal adhesion kinase? *BioEssays*, **17**, 229-236.

Ridley AJ. (1994). Membrane ruffling and signal transduction. *BioEssays*, **16**, 321-327.

Ridley AJ and Hall A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*, **70**, 389-399.

Rodriguez Fernandez JL, Geiger B, Salomon D and Ben-Ze'ev A. (1993).Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility, and anchorage-dependent growth of 3T3 cells. *J. Cell Biol.*, **122**, 1285-1294.

Rohrschneider LR. (1980). Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product. *Proc. Natl. Acad. Sci.*, **77**, 3514-3518.

Rohrschneider LR and Najita LM. (1984). Detection of the v-abl gene product at cell-substratum contact sites in Ableson murine leukemia virus-transformed fibroblasts. *J. Virol.*, **51**, 547-552.

Rosales C, O'Brien V, Kornberg L and Juliano R. (1995). Signal transduction by cell adhesion receptors. *Biochim. Biophys. Acta*, **1242**, 77-98.

Roskelley CD, Srebrow A and Bissell MJ. (1995). A hierarchy of ECM-mediated signalling regulates tissue specific gene expression. *Curr. Opin. Cell Biol.*,

7, 736-747.

Rozengurt E. (1995). Convergent signalling in the action of integrins, neuropeptides, growth factors and oncogenes. *Cancer Surveys*, **24**, 81-96.

Rudy W, Hofmann M, Schwartz-Albiez R, Zoller M, Heider KH, Ponta H and Herrlich P. (1993). The two major CD44 proteins expressed on a metastatic rat tumor cell line are derived from different splice variants: Each one individulally suffices to confer metastatic behavior. *Cancer Res*, **53**, 1262-1268.

Ruoslahti E and Reed JC. (1994). Anchorage dependence, integrins, and apoptosis. *Cell*, **77**, 477-478.

Saiki I, Murata J, Ida J, Nishi N, Sigimura K and Azuma I. (1989). The inhibition of murine lung metastasis by synthetic polypeptides [poly (arg-gly-asp) and poly (tyr-ile-gly-ser-arg)] with a core sequence of cell adhesion molecules. *Br. J. Cancer*, **59**, 194-197.

St. John T, Meyer J, Idzerda R and Gallatin WM. (1990). Expression of CD44 confers a new adhesive phenotype on transfected cells. *Cell*, **60**, 45-52.

Samuel SK, Hurta RAR, Spearman MA, Wright JA, Turley EA and Greenberg AH. (1993). TGF-β_1 stimulation of cell locomotion utilizes the hyaluronan receptor RHAMM and hyaluronan. *J. Cell Biol.*, **123**, 749-758.

Savani RC and Turley EA. (1995). The role of hyaluronan and its receptors in restenosis after balloon angiplasty: Development of a potential therapy. *Int. J. Tiss. Reac.*, **17**, 141-151.

Savani RC, Wang C, Yang B, Zhang S, Kinsella MG, Wight TN, Stern R, Nance DM and Turley EA. (1995). Migration of bovine aortic smooth muscle cells after wouding injury. The role of hyaluronan and RHAMM. *J. Clin. Invest.*, **95**, 1158-1168.

Schafer DA and Cooper JA. (1995). Control of actin assembly at filament ends. *Annu. Rev. Cell Dev. Biol.*, **11**, 497-518.

Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB and Parsons JT. (1992). pp125^{FAK}, a structurally unique protein tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci.*, **89**, 5192-5196.

Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR and Parsons JT. (1994). Autophosphorylation of the focal adhesion kinase, pp125^{FAK}, directs SH2-dependent binding of pp60^{src}. *Mol. Cell. Biol.*, **14**, 1680-1688.

Schaller MD and Parsons JT. (1994). Focal adhesion kinase and associated proteins. *Curr. Opin. Cell Biol.*, **6**, 705-710.

Schlaepfer DD, Hanks SK, Hunter T and vanderGeer P. (1994). Integrin-mediated signal transduction linked to ras pathway by GRB2 binding to focal adhesion kinase. *Nature*, **372**, 786-791.

Schreiner C, Fisher M, Hussein D and Juliano RL. (1991). Increased tumorigenicity of fibronectin receptor deficient Chinese hamster ovary variants. *Cancer Res*, **51**, 1738-1740.

Schuppan D and Hahn EG. (1987). Components of the extracellular matrix (collagens, elastin, glycoproteins, and proteoglycans). In *Mesechymal-*

Epithelial Interactions in Neural Development. Wolff JR, Berry M and Seivers J, (eds). Springer, Berlin. pp 3-29.

Schuppan D, Somasundaram R, Dietrich W, Ehnis T and Bauer M. (1994). The extracellular matix in cellular proliferation and differentiation. *Ann. NY Acad. Sci.*, **733**, 87-102.

Schwartz MA and Ingber DE. (1994). Integrating with integrins. *Mol. Biol. Cell*, **5**, 389-393.

Schwartz MA, Schaller MD and Ginsberg MH. (1995). Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Dev. Biol.*, **11**, 549-599.

Seftor REB, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E and Hendrix MJC. (1992). Role of the alpha v/beta 3 integrin in human melanoma cell invasion. *Proc. Natl. Acad. Sci.*, **89**, 1557-1561.

Seiter S, Arch R, Komitowski D, Hofmann M, Ponta H, Herrlich P, Matzhu S and Zoller M. (1993). Prevention of tumor metastasis formation by anti-variant CD44. *J. Exp. Med.* **177**, 443-455.

Sherman L, Sleeman J, Herrlich P and Ponta H. (1994). Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. *Curr. Opin. Cell Biol.*, **6**, 726-733.

Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BG, Birge RB, Fajardo JE, Chou MM, Hanafusa H, Schaffhausen B and Cantley LC. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell*, **72**, 767-778.

Spicer AP, Roller ML, Camper SA, McPherson JD, Wasmuth JJ, Hakim S, Wang C, Turley EA and McDondald JA. (1995). The human and mouse receptors for hyaluroanan-mediated motility, RHAMM, gene (*HMMR*) map to human chromosome 5q33.2-qter and mouse chromosone 11. *Genomics*, **30**, 115-117.

Starkey JR. (1990). Cell-matrix interactions during tumor invasion. *Cancer Metastasis Rev.*, **9**, 113-123.

Stetler-Stevenson WG, Aznavoorian S and Liotta LA. (1993). Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.*, **9**, 541-573.

Stoker M, O'Neill C, Perryman S and Waxmann V. (1983). Anchorage and growth regulation in normal and viral transformed cells. *Int. J. Cancer*, **3**, 683.

Stossel TP. (1993). On the crawling of animal cells. *Science*, **260**, 1086-1094.

Sy MS, Guo YJ and Stamenkovic I. (1991). Distinct effects of two CD44 isoforms on tumor growth *in vivo*. *J. Exp. Med.*, **174**, 859-866.

Sy MS, Guo YJ and Stamenkovic I. (1992). Inhibition of tumor growth *in vivo* with a soluble CD44-immunoglobulin fusion protein. *J. Exp. Med.*, **176**, 623-627.

Teder P, Bergh J and Heldin P. (1995). Functional hyaluronan receptors are expressed on a small cell lung carcinoma cell line but not on other lung carcinoma cell lines. *Cancer Res.*, **55**, 3908-3914.

Thomas L, Byers HR, Vink J and Stamenkovic I. (1992). CD44H regulates tumor cell migration on hyaluronate-coated substrate. *J. Cell Biol.*, **118**, 971-977.

Toyama-Sorimachi N, Sorimachi H, Tobita Y, Kitamura F, Yagita H, Suzuki K and

Miyasaka M. (1995). A novel ligand for CD44 is Serglycin, a hematopoietic cell lineage-specific proteoglycan. *J. Biol. Chem.*, **270**, 7437-7444.

Tsukita S, Oishi K, Sato N, Sagara J, Kawa IA and Tsukita S. (1994). ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J. Cell Biol.*, **126**, 391-401.

Turley EA. (1984). Proteoglycans and cell adhesion: their putative role during tumorigenesis. *Cancer Metastasis Rev.*, **3**, 325-329.

Turley EA. (1992). Hyaluronan and cell locomotion. *Cancer Metastasis Rev.*, **11**, 21-30.

Turley EA, Austen L, Vandeligt K and Clary C. (1991). Hyaluronan and a cell associated hyaluronan binding protein regulate the locomotion of *ras*-transformed cells. *J. Cell Biol.*, **112**, 1041-1047.

Turley EA, Belch AJ, Poppema S and Pilarski LM. (1993). Expression and function of a receptor for hyaluronan-mediated motility on normal and malignant B lymphocytes. *Blood*, **81**, 446-453.

Turley EA, Hossain MZ, Sorokan T, Jordan LM and Nagy JI. (1994). Astrocyte and microglia motility in vitro is functionally dependent on the hyaluronan receptor RHAMM. *Glia*, **12**, 68-80.

Turley EA, Tretiak M and Tanguay K. (1987) Effect of glycosaminoglycans and enzymes on the integrity of human placental aminion as a barrier to cell invasion. *J. Natl. Cancer Inst.*, **78**, 787-795.

Underhill CB. (1992) CD44: the hyaluronate receptor. *J. Cell Sci.*, **103**, 293-298.

Underhill CB, Green SJ, Comoglio PM and Tarone G. (1987). The hyaluronate receptor is identical to a glycoprotein of Mr 85,000 (gp85) as shown by a monoclonal antibody that interferes with binding activity. *J. Biol. Chem.*, **262**, 13142-13146.

Underhill CB and Toole BP. (1979). Binding of hyaluronate to the surface of cultured cells. *J. Cell Biol.*, **82**, 475-484.

Wang C, Entwistle J, Hou G, Li Q and Turley EA. (1996). The characterization of a human *RHAMM* cDNA: conservation of the hyaluronan-binding domains. *Gene*, **174**, 299-306.

Wang C and Turley EA. (1994). RHAMM is required for the motility and invasion of human breast cancer cell in vitro. (Abstract). *J. Cell. Biochem.*, Suppl. **18D**, 240.

Wang N, Butler JP and Ingber DE. (1993). Mechanotransduction across the cell surface and through the cytoskeleton. *Science*, **260**, 1124-1127.

Wei Y, Lukashev M, Simon DI, Bodary SC, Rosenburg S, Doyle MV and Chapman HA. (1996). Regulation of integrin function by the urokinase receptor. *Science*, **273**, 1551-1555.

Woods A and Couchman JR. (1988). Focal adhesions and cell-matrix interactions. *Collagen Rel. Res.*, **8**, 155-182.

Woods A and Couchman JR. (1992). Protein kinase C involvement in focal adhesion formation. *J. Cell Sci.*, **101**, 277-290.

Woods A and Couchman JR. (1994). Syndecan 4 heparan sulfate proteoglycan is a

selectively enriches and widespread focal adhesion component. *Mol. Biol. Cell*, **5**, 183-192.

Yang B, Zhang L and Turley EA. (1993). Indentification of two hyaluronan binding domains in the hyaluronan receptor RHAMM. *J. Biol. Chem.*, **268**, 8617-8623.

Yang B, Yang BL, Savani RC and Turley EA. (1994). Identification of a common hyaluronan binding motif in the hyaluronan binding proteins RHAMM, CD44 and link protein. *EMBO J.*, **13**, 286-296.

Zachary I and Rozengurt E. (1992). Focal adhesion kinase ($p125^{FAK}$): a point of convergence in the action of neuropeptide, integrins and oncogenes. *Cell*, **71**, 891-894.

Zetter BR. (1990). The cellular basis of site specific tumour metastasis. *New Engl. J. Med.*, **322**, 605-612.

Zigmond SH. (1996). Signal transduction and actin filament organization. *Curr. Opin. Cell Biol.*, **8**, 66-73.

Chapters:

Experimentation,

Results and

Discussion

Chapter 1

Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity

C.L. Hall, C. Wang, L.A. Lange and E.A. Turley

The Journal of Cell Biology,

Volume 126, Number 2, July 1994 , pages 575-588

Preface

The goal of this first chapter is to identify some of the signal transduction pathways and cytoskeletal changes that are required for HA:RHAMM induced cell motility. To approach this problem, we have chosen *ras*-transformed fibrosarcoma cells as the model system of study for the following reasons. First, previous studies have shown that rapid cell locomotion in *ras*-transformed fibroblasts results from the autocrine production of HA and the expression of the RHAMM receptor (Turley *et al.*, 1991; Hardwick *et al.*, 1992). Addition of either anti-RHAMM blocking antibodies or hyaluronan binding proteins significantly inhibits the motility of these cells. Second, RHAMM and *ras* have been shown to co-localize in lamellar extensions and membrane ruffles (Turley & Auersperg, 1989), structures typical of motile cells (see Stossel, 1993). This localization places RHAMM and *ras* in an ideal position to

regulate the cytoskeletal alterations required for elevated cell locomotion. Finally, we have chosen the *ras*-transformed cells as a model to gain insight into the mechanism of cell motility in tumor cells. Mutations of *ras* are present in a large population of human tumors (Bos, 1989) and cell motility is involved in invasion and metastasis of tumors (see Aznavoorian *et al.*, 1993).

After choosing a model system, we examined the role of tyrosine phosphorylation in HA:RHAMM signaling, as RHAMM associates with a protein complex that contains protein tyrosine kinase activity (Turley, 1989). Addition of HA to this complex *in vitro* stimulates tyrosine phosphorylation, suggesting that this event may be an early signal for HA:RHAMM induced cell motility *in vivo*. Moreover, tyrosine phosphorylation was beginning to emerge as an important regulator of focal adhesion formation and believed to be a critical step in the process of cell locomotion (see Clark & Brugge, 1995; Huttenlocher *et al.*, 1995; Lauffenburger & Horwitz, 1996). Of particular notoriety at sites of adhesion is the focal adhesion tyrosine kinase $pp125^{FAK}$. FAK plays a central role in adhesion formation and represents a point of convergence in signaling pathways regulated by the extracellular matrix, growth factors, neuropeptides and oncogenes (Zachary & Rozengurt, 1992; Rozengurt, 1995). We hypothesized that tyrosine phosphorylation of focal adhesion proteins, FAK specifically, is required for HA:RHAMM induced cell migration. Thus, in this chapter we utilize *ras*-transformed fibroblasts to examine the role of tyrosine phosphorylation and focal adhesion turnover in HA:RHAMM mediated cell locomotion.

Specific Objectives

1. To establish a model system in *ras*-transformed cells in order to study HA:RHAMM mediated cell locomotion, accompanying signaling pathways and cytoskeletal alterations.
2. To determine if tyrosine phosphorylation is required for HA:RHAMM mediated cell locomotion.
3. To determine the time course and the number of protein targets in the HA:RHAMM induced tyrosine phosphorylation pathway.
4. To determine the cellular localization of the tyrosine phosphorylation and the impact of phosphorylation on the cytoskeleton, specifically focal adhesions.
5. To identify specific focal adhesion targets of HA:RHAMM induced tyrosine phosphorylation.

Contribution

This chapter was published in the Journal of Cell Biology as referenced above. As a statement of my contribution to this work, all of the experiments shown (with the exception of the microinjection experiments done by LAL), the compilation of data, the research of materials and background literature and the writing of the manuscript were conducted by myself (CLH) under the guidance of EAT. Preliminary studies on FAK immunoprecipitation and focal adhesions immunofluorescent staining were done by CW.

References

Aznavoorian S, Murphy AN, Stetler-Stevenson WG and Liotta LA. (1993). Molecular aspects of tumor cell invasion and metastasis. *Cancer*, **71**, 1368-1383.

Bos JL. (1989). Ras oncogenes in human cancer: A review. *Cancer Res.*, **49**, 4682-4689.

Clark EA and Brugge JS. (1995). Integrins and signal transduction pathways: the road taken. *Science*, **268**, 233-239.

Hardwick C, Hoare K, Owens R, Hohn HP, Hook M, Moore D, Cripps V, Austen L, Nance DM and Turley EA. (1992). Molecular cloning of a novel hyaluronan receptor that mediated tumor cell motility. *J. Cell Biol.*, **117**, 1343-1350.

Huttenlocher A, Sandborg AA and Horwitz AF. (1995). Adhesion in cell migration. *Curr. Opin. Cell Biol.*, **7**, 697-706.

Lauffenburger DA and Horwitz AF. (1996). Cell Migration: A physically integrated molecular process. *Cell*, **84**, 359-369.

Stossel TP. (1993). On the crawling of animal cells. *Science*, **260**, 1086-1094.

Turley EA. (1989). Hyaluronic acid stimulates protein kinase activity in intact cells and in an isolated protein complex. *J. Biol. Chem.*, **264**, 8951-8955.

Turley EA and Auersperg N. (1989). A hyaluronate binding protein transiently codistributes with p21k-ras in cultures cell lines. *Exp. Cell Res.*, **182**, 340-348.

Turley EA, Austen L, Vandeligt K and Clary C. (1991). Hyaluronan and a cell-associated hyaluronan binding protein regulate cell locomotion in ras-transformed cell. *J. Cell Biol.*, **112**, 1041-1047.

Zachary I and Rozengurt E. (1992). Focal adhesion kinase ($p125^{FAK}$): a point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell*, **71**, 891-894.

HYALURONAN AND THE HYALURONAN RECEPTOR RHAMM PROMOTE FOCAL ADHESION TURNOVER AND TRANSIENT TYROSINE KINASE ACTIVITY

Christine L. Hall, Chao Wang, Laurie A. Lange and Eva A. Turley

Departments of Pediatrics and Physiology, and

Manitoba Institute of Cell Biology

100 Olivia Street

Winnipeg, Manitoba

Canada R3E 0V9

Phone: (204) 787-2137

FAX: (204) 787-2190

Running Title: HA promoted locomotion requires PTK activity

ABSTRACT

The molecular mechanisms whereby hyaluronan (HA) stimulates cell motility were investigated in a C-H-*ras* transformed 10T½ fibroblast cell line (C3). A significant ($p < 0.001$) stimulation of C3 cell motility with HA (10 ng/ml) was accompanied by an increase in protein tyrosine phosphorylation as detected by anti-phosphotyrosine antibodies using immunoblot analysis and immunofluorescence staining of cells. Tyrosine phosphorylation of several proteins was found to be both rapid and transient with phosphorylation occurring within 1 min of HA addition and dissipating below control levels 10 - 15 min later. These responses were also elicited by an antibody generated against a peptide sequence within the HA receptor RHAMM. Treatment of cells with tyrosine kinase inhibitors (genistein, 10 μg/ml or herbimycin A, 0.5 μg/ml) or microinjection of anti-phosphotyrosine antibodies inhibited the transient protein tyrosine phosphorylation in response to HA as well as prevented HA stimulation of cell motility. To determine a link between HA-stimulated tyrosine phosphorylation and the resulting cell locomotion, cytoskeletal reorganization was examined in C3 cells plated on fibronectin and treated with HA or anti-RHAMM antibody. These agents caused a rapid assembly and disassembly of focal adhesions as revealed by immunofluorescent localization of vinculin. The time course with which HA and antibody induced focal adhesion turnover exactly paralleled the induction of transient protein tyrosine phosphorylation. In addition, phosphotyrosine staining co-localized with vinculin within structures in the lamellapodia of these cells. Notably, the focal adhesion kinase, $pp125^{FAK}$ was rapidly

phosphorylated and dephosphorylated following HA stimulation. These results suggest that HA stimulates locomotion via a rapid and transient protein tyrosine kinase signalling event mediated by RHAMM. They also provide a possible molecular basis for focal adhesion turnover, a process that is critical for cell locomotion.

INTRODUCTION

Components of the extracellular matrix (ECM) play a fundamental role in the process of cell migration and hyaluronan (HA), an ECM glycosaminoglycan, has been particularly well studied in this context. HA has been shown to promote cell motility *in vitro* (Bernanke and Markwald, 1979; Boudreau *et al.*, 1991; Hadden and Lewis, 1991; Hakansson *et al.*, 1980a & b; Hardwick *et al.*, 1992; Huszar *et al.*, 1990; Kornovski *et al.*, 1993; Savani *et al.*, submitted; Stamenkovic *et al.*, 1991; Turley *et al.*, 1991; West and Kumar, 1989) and to contribute to the motile responses of cells during wound repair (Toole *et al.*, 1984; Weigel *et al.*, 1986, 1989), tumor invasion (Iozzo, 1985; Pauli *et al.*, 1983; Toole, 1982; Turley, 1984) and tissue morphogenesis (Copp and Bernfield, 1988a & b; Toole *et al.*, 1984, 1989). Like other ECM components that bind to specific receptors on cells (Aznavoorian *et al.*, 1990; McCarthy *et al.*, 1985, 1986; Mensing *et al.*, 1985; Yusa *et al.*, 1989), HA interacts with cell surface receptors, which govern locomotion (reviewed by Turley, 1992). Thus, it has been shown that the HA receptor, RHAMM, mediates the motility of *ras*-transformed fibroblasts (Turley *et al.*, 1991; Hardwick *et al.*, 1992)

and that the HA receptor CD44 is associated with lymphocyte homing (Stamenkovic *et al.*, 1991) and tumor cell migration on HA substrata (Hart *et al.*, 1991; Thomas *et al.*, 1992). The molecular mechanisms underlying HA regulation of cell locomotion are unknown, but interactions between ECM elements and cell surface receptors have been linked to adhesion and signal transduction events that are required to initiate a motility response (reviewed in Lester and McCarthy, 1992; Ruoslahti, 1992; Starkey, 1990). Similarly, we have proposed (Turley, 1989a & b; Turley, 1992) that HA-receptor interactions trigger a signal transduction cascade that is responsible for orchestrating key features of locomoting cells, namely lamellae formation and ruffling (unpublished results; Abercrombie *et al.*, 1970, 1977; Goldman *et al.*, 1976; Harris, 1973; Izzard and Lochner, 1980).

The formation of cellular protrusions in the course of cell locomotion involves reorganization of actin networks at the leading edge of cells and constant formation/disassembly of close contacts and focal adhesions at the cell margin (Burridge *et al.*, 1988). Although formation of focal adhesions is necessary for cell locomotion, these structures when present in a stable state appear to impede locomotion and there are fewer, less developed focal adhesions in motile cells. Focal adhesions may also harbour a composite of signal transduction mechanisms such that they may receive information from the ECM and serve as a point of convergence for signals from other pathways (see Gingell and Owens, 1992; Lo and Chen, 1994; Zachary and Rozengurt, 1992). This possibility is consistent with the presence of regulatory molecules such as protein kinase C (Jaken *et al.*, 1989; Woods and

Couchman, 1992), a calcium-dependant protease (Beckerle *et al.*, 1987) and tyrosine kinase oncoprotein (Rohrschneider, 1980; Rohrschneider and Gentry, 1984; Rohrschneider and Najita, 1984) in focal adhesions as well as with the post-transcriptional modifications these structures have been reported to undergo (Beckerle, 1990; Declue and Martin, 1987; Freed *et al.*, 1989; Glenney and Zokas, 1989; Hirst *et al.*, 1986; Litchfield and Ball, 1986; Pasquale *et al.*, 1986; Sefton *et al.*, 1981; Shaw *et al.*, 1990; Tapley *et al.*, 1989; Turner *et al.*, 1989; Werth *et al.*,1983; Werth and Pastan, 1984). Furthermore, growth factors and tumor promoters affect focal adhesion organization (reviewed in Burridge *et al.*, 1988) and perhaps most interestingly, a specific focal adhesion tyrosine kinase (FAK), $pp125^{FAK}$ (Schaller *et al.*, 1992) has been identified. In view of the forgoing base of information, identification of the factors that regulate focal adhesion assembly/ disassembly and the characterization of the molecular sequelae of events leading to their turnover will likely contribute substantially to understanding of mechanisms whereby diverse stimuli promote cell locomotion.

In this study, C-H-*ras*-transformed 10T½ fibroblasts were utilized as a model of metastatic cells to investigate signal transduction processes in response to HA-induced locomotion, since these cells utilize HA to maintain a highly motile phenotype (Turley *et al.*, 1991). We demonstrate that HA initiates locomotion in these cells via a protein tyrosine phosphorylation pathway, that HA regulates focal adhesion turnover, and that these effects are mediated by the HA receptor RHAMM. Our results provide the first direct evidence for the proposal that ECM-cell

interactions evoke rapid intracellular signals that directly lead to locomotion. We suggest that focal adhesion turnover is an immediate result of the HA signal transduction pathway and that regulation of this pathway by HA is one of the events essential for cell locomotion to occur.

MATERIALS AND METHODS

Cell Culture

As previously described (Egan *et al.*, 1987), the CIRAS-3 (C3) cell line is derived from a 10T½ cell line transfected with the H-*ras* and *neo*^{R} genes. The cells were maintained at 37°C in 5% CO_2 on plastic tissue culture dishes (Corning, Corning, NY) in alpha-MEM growth media (Gibco BRL, Grand Island, NY) supplemented with 10 mM HEPES, pH 7.4 (Sigma Chemical Co., St. Louis, MO) and 10% FCS (Hyclone Laboratories, Inc., Logan, Utah). Cells were subcultured in 0.25% trypsin (Difco Bactotrypsin)/2 mM EDTA (Sigma) when cultures reached 80% confluence. All experiments were performed on cells with low passage numbers (P9 - P15) and at 24 h after subculture, a time when cells were locomoting rapidly and expressing elevated levels of HA receptors (data not shown). For motility studies, 10^5 cells were placed into 25 cm^2 tissue culture flasks (Corning) containing growth media and maintained as above. After 12 h of growth, the media was aspirated, the cells were rinsed with HBSS (Gibco BRL), and fresh serum-free medium containing 4.0 μg/ml transferrin (human, Gibco BRL) and 2.0 μg/ml insulin (bovine, Sigma) was added to the flasks. The cells were maintained in this defined

medium for another 12 h after which the media was changed to fresh defined media prior to the cell motility analysis. Cells were treated as above for all remaining experiments except as follows: for phosphorylation and immunoprecipitation analysis, 5 x 10^5 cells were plated on 100 mm tissue culture dishes (Corning); for immunofluorescence, 10^5 cells were plated on sterile coverslips in 60 mm dishes; and for microinjection, 10^5 cells were plated on 60 mm dishes. To maintain cells at physiological pH during microinjection, an altered defined media containing 25 mM HEPES (instead of 10 mM HEPES) and 1.8 g/L $NaHCO_3$ (instead of 3.6 g/L $NaHCO_3$) was added prior to injection. In cases where cells were plated on a fibronectin coated surface, coverslips or culture dishes were incubated with human plasma fibronectin (50 μg/ml; Collaborative Biomedical/Becton Dickinson Labware, Bedford MA) overnight at 4°C prior to plating of cells.

Addition of HA and Antibody

Varying dilutions of Healon® (0.1 ng/ml - 1 μg/ml; rooster comb hyaluronan; Pharmacia LKB Biotechnology, Uppsala, Sweden) were added directly to the culture medium of C3 cells in order to test appropriate concentrations required to stimulate cell motility. A concentration of 10 ng/ml was found to be optimal and was used in all HA stimulation experiments. Similarly, antisera generated against a peptide corresponding to a sequence in RHAMM (amino acids 125-145, QEKYNDTAQSLRDVTAQLESV; Hardwick *et al.*, 1992) was added to C3 fibroblasts and a 1/100,000 dilution was found to be optimal for stimulating locomotion. The same dilution of pre-immune serum was used as control.

Treatment of C3 Fibroblasts with Tyrosine Kinase Inhibitors

Stock solutions of genistein and herbimycin A (Gibco BRL) were prepared in DMSO and stored at -20°C. Concentrations of 10 μg/ml (37 μM) of genistein or 0.5 μg/ml (0.87 μM) of herbimycin A (Akiyama *et al.*, 1987; Uehara *et al.*, 1989) were added directly to the cell culture medium. Timed studies indicated that a pretreatment time of 10 min for genistein or 4 h for herbimycin A was required to inhibit the HA-induced locomotory responses (data not shown). For controls, cells were treated with DMSO for the same time periods.

Locomotion Analysis

Cell locomotion was recorded using a computerized time-lapse image analysis system (Image-1, Universal Imaging Corporation, Westchester, PA) capable of measuring random locomotion by nuclear displacement. During the filming period, cells were maintained in defined medium (described above) at physiological pH on a heated stage (37°C). For each experiment, a minimum of 30 cells were tracked either every 20 min for a 10 h period or every 10 min for a 1-2 h period. At least 3 trials of each experimental group were observed for a total of 90 cells per group. The results were expressed as means (μm/h) $\pm$ SEM.

Microinjection

Twenty-four hours after subculture, C3 fibroblasts, prepared as described above, were microinjected with anti-phosphotyrosine antibody [rabbit polyclonal; Upstate Biotechnology Incorporated (UBI), Lake Placid, NY] or control antibody (rabbit IgG; Sigma). Antibodies (2 mg/ml) were dissolved in injection buffer (150

mM KCl, 2 mM MOPS, pH 7.0; Cooper *et al.*, 1988) containing 1 mg/ml BSA and Lucifer yellow dye, then backloaded into micropipettes (pulled to 1 μm tip diameter using a model PD-5 microelectrode puller; Narishige Scientific Instrument Lab., Tokyo, Japan). Cells were microinjected using an Eppendorf microinjector (model 5242; Eppendorf, Hamburg, Germany) and Leica micromanipulator on a Zeiss fluorescence microscope. After microinjection, cells were allowed to recover for 30 min before addition of HA and commencement of motility analysis.

Cell Lysis and Immunoblot Analysis

C3 cell cultures were exposed to HA (10 ng/ml), anti-RHAMM, or control treatments for various time periods at 37°C then placed on ice. The culture media was removed, the plates rinsed with cold PBS (2.7 mM KCL, 1.1 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4; pH 7.4) containing 250 μM sodium orthovanadate and the cells were lysed with ice cold RIPA lysis buffer (25 mM tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA) containing 1 μM leupeptin, 1000 KU/ml Aprotinin, 1 μg/ ml PMSF and 1 mM sodium orthovanadate (all chemicals from Sigma). Lysates were scraped into microcentrifuge tubes and after 10 min on ice were centrifuged at 13,000 rpm for 15 min at 4°C (Heraeus Biofuge 13, Baxter Diagnostics Corporation, Mississauga, Ontario). Protein concentrations of the supernatants and BSA standards were determined using the *DC* protein assay (Bio-rad Laboratories, Rockville Center, NY) and duplicate samples containing 100 μg of protein each along with prestained molecular weight markers (Sigma) were separated by SDS-PAGE (10% gel;

Laemmli, 1970). The proteins on the gels were either electrophoretically transferred to nitrocellulose membranes (Bio-rad) or stained with Coomassie blue to check for equal loading. Additional protein binding sites on the nitrocellulose membranes were blocked with 5% defatted milk in TBS (50 mM Tris HCl, pH 7.4, 200 mM NaCl) then the membranes were incubated with anti-phosphotyrosine MAb [1 μg/ml 4G10 (UBI) in 1% defatted milk/TBS] for 2 hours at room temperature on a rotator (Nutator; Becton, Dickinson and Company, Parsippany, NJ). The membranes were washed 4 times in 0.05% Tween-20/TBS before incubation with peroxidase-conjugated goat anti-mouse secondary antibody (1:5000 dilution in 1% milk/Tween-TBS; Sigma) for 1 h at room temperature. After washing, blots were developed using the ECL Western blotting detection system (Amersham International plc, Amersham, UK) according to manufacturers instructions. To establish antibody specificity, parallel blots were probed with anti-phosphotyrosine that had been pre-incubated with 200 μM phosphotyrosine (Sigma) for 1 h. To further check for equal loading, blots were stripped (stripping buffer: 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, at 50° for 30 min), blocked and reprobed using an anti-vinculin MAb [1:200; Sigma] (data not shown). Densitometry of the blots was performed on a Bio-rad Video Densitometer (Model 620) and analyzed using the 1-D Analyst II software.

Immunoprecipitation

C3 fibroblasts were prepared, stimulated with HA and lysed as above. Each sample (500 μg of protein) was incubated with anti-p125^{FAK} MAb (10 μg/ml; UBI)

and goat anti-mouse IgG (5 μg/ml; Sigma) for 1 h at 4°C by mixing end-over-end. To precipitate, 100 μl Protein G-Agarose (Gibco BRL) was added to each tube and the samples were mixed end-over-end for another 30 min at 4°C. The beads were pelleted by brief centrifugation at 13,000 rpm and washed 3 times with RIPA buffer. The proteins were released from the beads by boiling in Laemmli sample buffer then subjected to SDS-PAGE and anti-phosphotyrosine immunoblotting as above. To assess whether equal quantities of FAK were immunoprecipitated from control and treated samples, the blots were stripped and reprobed with purified anti-FAK (10 μg/ml antibody 2A7; a generous gift from J.T. Parsons, University of Virginia, Charlottesville, VA).

Immunofluorescent Staining

C3 fibroblasts were grown on untreated or fibronectin coated glass coverslips for 24 h as described above. The cells were exposed to HA (10 ng/ml), anti-RHAMM or control treatment for 1 min, 5 min, 15 min, 30 min or 1 h. At the appropriate time periods, the media was aspirated, the cells rinsed with PBS, then fixed with 3% paraformaldehyde (Sigma)/PBS for 10 min. Cells were washed 3 times for 10 min with wash solution (10% FCS/PBS containing 0.02% sodium azide), permeabilized with 0.2% Triton X-100/PBS for 5 min, and washed 3 more times. For single immunofluorescence studies, the fixed cells were incubated either at 4°C overnight or at 37°C for 2 h with anti-phosphotyrosine MAb (5 μg/ml; 4G10, UBI), anti-vinculin MAb (1:50; Sigma), or appropriate IgG controls (Sigma) in wash solution. After washing 5 times, coverslips were incubated with goat anti-mouse

TRITC (1:300; Sigma) for 3 h. For double immunofluorescence studies, the cells were simultaneously incubated with anti-vinculin and a rabbit polyclonal anti-phosphotyrosine (10 μg/ml; UBI) primary antibodies and with goat anti-mouse TRITC and goat anti-rabbit FITC secondary antibodies as above. After washing, the coverslips were mounted in no-fade mountant (10 mM p-phenylenediamine, 118 mM Tris-HCl, pH 7.4, 90% glycerol) onto glass slides and sealed with nail polish. Observations and photomicrographs were obtained with a Zeiss Axiovert 35M fluorescent microscope using epifluorescence.

RESULTS

Exogenous HA Stimulates Locomotion of C3 Fibroblasts

HA has previously been reported to stimulate the locomotion of *ras*-transformed fibroblasts (Hardwick *et al.*, 1992; Turley *et al.*, 1991). Similarly, the response of the *ras*-transformed 10T½ cell line (Egan *et al.*, 1987) utilized here (C3 cells) was presently found to be concentration dependent (data not shown) with maximal stimulation of motility occurring at 10 ng/ml of HA. Two hours after addition of HA, C3 cells reached a peak locomotion rate of 18.8 μm/h, which was significantly different ($p < 0.001$) from that of control untreated cells (Fig. 1).

Exogenous HA Stimulates Tyrosine Phosphorylation in C3 Fibroblasts

C3 cells respond to HA with an increase in protein tyrosine phosphorylation as determined by immunoblot analysis (Fig. 2) and immunofluorescent staining (Fig. 3) with anti-phosphotyrosine antibodies. The phosphorylation response was both

rapid and transient; it occurred within 1 min of HA addition (10 ng/ml) and decreased within the subsequent 5-15 min (Figs. 2,3). In Figure 2*A*, multiple protein bands (185, 125, 115, 85, 75, 65, 60, 56, 50, 40 and 38 kDa) in cell lysates are seen to be tyrosine phosphorylated and many of these show a transient increase over controls following 1 min of HA stimulation (Fig. 2*B*). As well, after 15 min of HA stimulation (Fig. 2*A*, lane 5), the relative levels of tyrosine phosphorylated proteins drop below control levels (Fig. 2*A* and *B*) indicating that a secondary dephosphorylation event follows the initial phosphorylation. Anti-phosphotyrosine recognition of all protein bands above is completely eliminated by pre-incubation of the antibody with phosphotyrosine (data not shown). In addition, densitometric analysis of a Coomassie blue stained parallel gel shown in Figure 2*C* and *D* demonstrates equal loading of cell lysates.

Figure 3 (*a-c*) shows anti-phoshotyrosine immunofluorescence of representative control cells demonstrating the variations of staining observed in these experiments. Immunofluorescence revealed a transient increase and a distinct pattern of anti-phosphotyrosine antibody staining following HA stimulation (Fig. 3*d-f*). After 1 min of HA treatment, an increase in staining generally occurred along the advancing lamellae, that was often amorphous (Fig. 3*d*) or in punctate regions. As well, the punctate regions of staining become larger than seen in control cells (Fig. 3*a-c*). Although difficult to quantitate, in all experiments over 40% of the 1 min HA stimulated cells exhibited the high intensity of punctate staining observed in Fig. 3*d* while less than 5% of control treated cells showed a similar intensity of staining.

Consistent with Western blot analysis, staining decreased to below control levels by 15 min (Fig. 3*g,h*).

Anti-RHAMM Mimics HA Responses

Antibodies generated against RHAMM have previously been shown to block the motility of *ras*-transformed cells (Hardwick *et al.*, 1992; Turley *et al.*, 1991). However, low concentrations of an anti-peptide antibody to RHAMM was found here to mimic HA in that it stimulated both cell locomotion (Fig. 4*A*) and tyrosine phosphorylation (Fig. 4*B* and *C*). The time course and staining patterns observed in these experiments is similar to those seen above in response to HA; locomotion reached a maximum in the first 2 h after antibody addition and tyrosine phosphorylation peaked after 1 min, but decreased below control levels by 15 min after antibody stimulation. In Fig. 4*C*, representative cells were chosen to display the variations in staining patterns observed. As noted after HA stimulation, protein tyrosine phosphorylation occurred primarily in the leading lamellae as plaque-like and amorphous staining.

Tyrosine Kinase Inhibitors Block HA-Mediated Motility

To determine whether inhibitors of protein tyrosine kinases effectively block HA stimulated locomotion, the two inhibitors genistein and herbimycin A were utilized. Genistein (10 μg/ml), added to cell cultures 10 min prior to HA stimulation, abrogates the HA stimulated tyrosine phosphorylation (Fig. 5*A, B*). Both inhibitors block protein tyrosine phosphorylation as determined by Western blot analysis (Fig. 5*A, B*) and immunofluorescence (data not shown).

Addition of genistein to C3 fibroblasts had no affect on **basal** motility rate of control cells (Figs. 1 and 6), indicating that it is non-toxic. However, genistein pre-treatment of C3 cells prevents the HA-induced increase in motility (Fig. 6). Addition of herbimycin A (0.5 μg/ml; 4 h pre-treatment) to C3 cells similarly results in inhibition of HA-mediated motility (Fig. 7).

Cells were stimulated with HA just prior to the addition of genistein to determine whether an initial burst of protein tyrosine phosphorylation is sufficient for enhancement of cell locomotion by HA. This experimental paradigm does not inhibit a locomotory response to HA (Fig. 8), indicating that only subsequent protein tyrosine phosphorylation events are necessary for increased locomotion elicited by HA (Fig. 8).

Microinjection of Anti-phosphotyrosine Blocks HA-mediated Motility

To further assess the role of protein tyrosine phosphorylation in HA-mediated cell locomotion, C3 fibroblasts were microinjected with either an anti-phosphotyrosine polyclonal antibody or a control antibody. After recovery from injection for 30 min, cells were treated with HA and their motility was recorded for 2 h. The controls responded to HA with a significant ($p < 0.01$) increase in locomotion (injected, unstimulated control cells locomote at 10.4 ± 1.1) (Fig. 9*A, B*), while the motility of the anti-phosphotyrosine-injected cells was inhibited to below basal level (Fig. 9*C*).

HA Promotes Reorganization and Phosphorylation within Focal Adhesion Sites

Locomoting *ras*-transformed fibroblasts, like other motile cells, exhibit few

poorly developed focal adhesions (Takahashi *et al.*, 1986; Fig. 10) and display vinculin staining that localizes to heavily stained belts within the lamellae, to contact points in the trailing end of cells, and to punctate regions in the cell body and lamellapodia that do not resemble the more developed focal contacts seen in stationary normal fibroblasts. To facilitate the study of focal contacts in *ras*-transformed cells, C3 cells were plated on fibronectin coated surfaces which are known to promote focal adhesion assembly even in transformed cells (see Burridge *et al.*, 1988). Under these conditions, the cells exhibited a somewhat more flattened appearance, but remained responsive to HA and stimulatory anti-RHAMM antibody (data not shown). Anti-vinculin immunofluorescence staining of these cells plated on fibronectin (Fig. 10*a-c*) revealed the presence of mainly small punctate focal contacts as well as vinculin staining within the lamellae of cells. However, immediately following HA addition (1 min; Fig. 10*d-f*), the vinculin staining increased, appearing as well defined plaques within the lamellapodia and along the leading edge of the cells, typical of focal adhesions of less motile or normal cells. This appearance of focal adhesions is transient and by 15 min after HA addition (Fig. 10*g* and *h*) there is a loss of staining and cells begin to more closely resemble control cells. The rapid appearance/disappearance of distinct focal adhesions corresponds to the transient stimulation of HA-induced protein tyrosine phosphorylation. Cells treated with anti-RHAMM also exhibit this induced focal adhesion turnover in an identical time course (data not shown).

Double staining of the cells with anti-vinculin MAb and polyclonal anti-

phosphotyrosine Ab show that when cells are maintained under standard culture conditions without fibronectin, phosphotyrosine co-localizes with vinculin staining in the lamellae of locomoting cells (Fig. 11). Co-localization of vinculin and phosphotyrosine at the front of the cells suggests that tyrosine phosphorylation is associated with immature, newly formed adhesion sites at the leading lamellae of the cells. Similar results were obtained when cells were maintained on a fibronectin substrate.

Focal Adhesion Kinase, pp125^{FAK}, is Phosphorylated/Dephosphorylated with HA Stimulation

Because HA stimulation results in both an increase in the specific tyrosine phosphorylation of a 125 kDa protein (the size of FAK), since FAK is localized to focal adhesions, and since integrins have been shown to mediate an increase in FAK phosphorylation (Burridge *et al.*, 1992; Guan and Shalloway, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992; Lipfert *et al.*, 1992), we examined the effect of HA addition on the tyrosine phosphorylation of this kinase. In contrast to reports of integrin-activated cells, where a prolonged stimulation of FAK phosphorylation occurs (Burridge *et al.*, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992; Lipfert *et al.*, 1992), HA treatment results in only a transient increase in FAK phosphorylation (1 min; Fig. 12) and this is followed by a clear decrease in phosphorylation of this kinase (5 min; Fig. 12). The time course of FAK phosphorylation/dephosphorylation coincides with the HA stimulated assembly/disassembly of adhesion plaques within the C3 fibroblasts. Although this data supports a role for FAK in HA promoted focal

adhesion assembly/disassembly, the kinase could not be detected in the focal adhesions of these cells by immunofluorescent staining (data not shown).

DISCUSSION

HA is emerging as a critical regulator of the locomotion of fibroblasts (Bernanke and Markwald, 1979; Hardwick *et al.*, 1992; Turley *et al.*, 1991), epithelial cells (Hadden and Lewis, 1991), white cells (Hakansson *et al.*, 1980a & b; Stamenkovic *et al.*, 1991), smooth muscle cells (Boudreau *et al.*, 1991; Savani *et al.*, submitted), sperm (Huszar *et al.*, 1990; Kornovski *et al.*, 1993) and vascular endothelial cells (Banerjee and Toole, 1992; West and Kumar, 1989). Moreover, HA is required in the process whereby motility factors (Schor *et al.*, 1989) and growth factors such as TGF-β (Samuel *et al.*, 1993) regulate locomotion *in vitro*. Although virtually nothing is known about the molecular mechanisms underlying HA action on cell motility, its weakly adhesive physicochemical properties have been considered to contribute to cell detachment during locomotion. We provide evidence here that HA/receptor interactions, like ECM/integrin interactions (Juliano and Haskell, 1993), result in cell signaling events culminating in profound effects on cell behaviour. The C-H-*ras* fibrosarcoma (C3) cells are a rapidly locomoting cell line that requires HA/RHAMM interactions to maintain their highly motile phenotype. Here we show that HA initiates cell locomotion in C3 cells via a RHAMM-induced protein tyrosine kinase (PTK) pathway, that inhibition of this PTK activity abolishes the HA locomotory response, and that an initial, short-lasting phosphorylation event

is required for the stimulation of motility to occur. These results clearly implicate the regulation of protein tyrosine phosphorylation in ECM-driven cell locomotion. We also show that the transient assembly of focal adhesions results from HA signaling and identify HA as the first extracellular ligand to regulate focal adhesion turnover concomitant with enhanced locomotion. We further identify pp125^{FAK} as a protein target of the HA signaling pathway. Perhaps most importantly, and unlike the effect of other ECM components, the effect of HA on protein tyrosine phosphorylation is transient, such that the burst of tyrosine phosphorylation is rapidly dampened to below control levels, and focal adhesions disappear as rapidly as they appear. This transient event suggests that HA may also regulate phosphatases or perhaps other signal transduction pathways that are involved in focal adhesion disassembly.

The HA:RHAMM signaling pathway is clearly complex in nature and can be divided into two phases. In the first phase, HA:RHAMM interactions mediate both rapid and transient protein tyrosine phosphorylation and focal adhesion turnover, with the brief tyrosine phosphorylation and focal adhesion assembly (after 1 min of stimulation) giving way to a more long term dephosphorylation and disassembly (within 15 min of stimulation). Further, if HA is added prior to the inhibitor genistein, stimulation still occurs indicating that only the rapid and transient burst in tyrosine phosphorylation in the first phase is sufficient for stimulating the subsequent motility. This first phase may be critical for bringing together regulatory proteins within focal adhesion sites. In the second phase, cell locomotion increases significantly and peaks within the first 2 h of HA or anti-RHAMM stimulation.

Thus, elevated cell motility correlates with protein tyrosine dephosphorylation and focal adhesion disassembly. This appears initially to be at odds with our observation that treatment of fibroblasts with tyrosine kinase inhibitors, genistein or herbimycin A, or with microinjected anti-phosphotyrosine antibodies inhibits HA mediated motility. Since the inhibitors did not further enhance the locomotory/ dephosphorylation phase, as one might expect, the initial organization of focal adhesions and concomitant protein tyrosine phosphorylation must be required for subsequent locomotory events. The molecular basis for this requirement is not yet clear. As a result, there is an interesting turn of events in which an initial burst of tyrosine phosphorylation triggers a later cell motility/dephosphorylation phase.

Such an association between protein tyrosine phosphorylation and downstream locomotory behaviour has been noted previously. PTK activity has been implicated in the ligand-induced membrane ruffling of human epidermoid carcinoma cells (Izumi *et al.*, 1988) and in neutrophil migration induced by the chemotactic factor fMet-Leu-Phe (Gaudrey *et al.*, 1992). As well, other chemotactic factors and ECM molecules that regulate cell locomotion also promote protein phosphorylation (Bottaro *et al.*, 1991; Ferrel and Martin, 1989; Mueller *et al.*, 1992; Nakamura and Yamamura, 1989; Segall and Gerisch, 1989; Zigmund, 1989). In particular, the cell motility triggered by scatter factor is mediated by the tyrosine kinase receptor c-Met (Komada and Kiamura, 1993; Weidner *et al.*, 1993). Like HA:RHAMM signaling, c-Met activation and tyrosine phosphorylation results in cell motility that occurs more downstream. Although these studies predict that protein phosphorylation is

essential for ligand-induced locomotion, the targets of these protein kinase cascades have remained elusive. It is likely that ligand binding and signal transduction, such as ligand regulated PTKs, target the cytoskeleton and result in changes in cytoskeletal organization which is necessary for cell locomotion. Since focal adhesion turnover is required for cell locomotion, these adhesion sites are also likely targets for factors that stimulate locomotion. Cycles of attachment and detachment, which are a prerequisite to lamellae extension and hence cell motility, are likely to be mediated by close contacts and point focal adhesions (Burridge *et al.*, 1988). Excessive development of mature focal adhesions, regulated in part by ECM/integrin interactions (see Burridge *et al.*, 1988), impedes locomotion. On the other hand, factors that promote extensive focal adhesion disassembly such as tenascin also inhibit locomotion (Chiquet-Ehrismann *et al.*, 1988; Lightner and Erickson, 1990; Murphy-Ullrich *et al.*, 1991; Sage and Bornstein, 1991). In contrast, factors like HA that promote rapid turnover of focal adhesions allow for rapid locomotion. Strong detaching mechanisms provided by tenascin may be necessary for rear detachment, while a more subtle detaching/attaching mechanism under the influence of HA may be required for lamellae extension.

Reports indicate that focal adhesion structure and function are regulated by changes in protein tyrosine phosphorylation (Burridge *et al.*, 1992; Guan and Shalloway, 1992; Hanks *et al.*, 1992; Schaller *et al.*, 1992) and that these structures contain tyrosine phosphorylation sites in both normal (Maher *et al.*, 1985) and transformed cells (Comoglio *et al.*, 1984). In addition, focal adhesion disassembly

is promoted by TPA (Schliwa *et al.*, 1984), which activates protein kinase C, and some viral oncogenes, while their assembly has been correlated with localized protein tyrosine phosphorylation in response to ECM/integrin interactions (Burridge *et al.*, 1992; Guan *et al.*, 1991; Kornberg *et al.*, 1991). These observations in conjunction with our results support a role for tyrosine phosphorylation in the assembly and maintenance of focal adhesions in spreading and stationary cells, as well as a role for transient phosphorylation in initiating focal adhesion turnover required for cell locomotion. A potentially important regulator of focal adhesions and, as shown here a target for HA-induced signaling, is the newly characterized focal adhesion kinase. Tyrosine phosphorylation of FAK in response to extracellular matrix proteins has been associated with increased kinase activity, cell adhesion and focal adhesion assembly (reviewed in Zachary and Rozengurt, 1992). The coincidental occurrence noted here of tyrosine phosphorylation of FAK and focal adhesion assembly, as well as the dephosphorylation of FAK coincident with the loss of focal adhesions in response to HA, is consistent with a role for this protein in focal adhesion turnover in HA signaling as well. However, the possibility that FAK plays a passive role in HA signaling cannot at this point be ruled out.

Although RHAMM possesses no intrinsic kinase activity, a complex associated with RHAMM contains an HA-stimulated PTK (Turley, 1989a) that is antigenically related to $pp60^{c\text{-}src}$ (Turley, 1989b). In addition, RHAMM also co-localizes both with cytoskeletal elements on locomoting chick heart fibroblasts (Turley *et al.*, 1990) and with $pp60^{c\text{-}src}$ on mouse fibroblasts (unpublished

observation). Since members of the src family kinases are known to associate with several membrane proteins (see Bolen *et al.*, 1991 and references therein), it is possible that the src kinase, or another member of the src family, could be associated with RHAMM and thus be involved in the HA triggered tyrosine phosphorylation. In this light, connections between src family kinases, the cytoskeleton and cell motility have been reported; $pp60^{v\text{-}src}$ has been found to be associated with cytoskeletal proteins (Hamaguchi and Hanafusa, 1987), adhesion plaques and focal adhesions (Nigg *et al.*, 1982; Rohrschneider, 1980; Rohrschneider *et al.*, 1982; Shriver and Rohrschneider, 1981a & b). As well, overexpression of c-*src* in endothelial cells increases their rate of locomotion (Bell *et al.*, 1992). Activated src substrates include several cytoskeletal or cytoskeletal-associated proteins as well as $pp125^{FAK}$ (Davis *et al.*, 1991; Glenney and Zokas, 1989; Kanner *et al.*, 1991a & b; Matten *et al.*, 1990; Pasquale *et al.*, 1986; Powell and Glenney, 1987; Reynolds *et al.*, 1992; Rothberg *et al.*, 1992; Schaller *et al.*, 1992; Sefton *et al.*, 1981; Turner *et al.*, 1990; Wu and Parsons, 1993). In fact, among the tyrosine phosphorylated *src* substrates, p130, p120, p110, p210, p125, p118, p85, p185/p64 (Kanner *et al.*, 1990), six have similar molecular weights to the presently observed proteins phosphorylated in response to HA stimulation. Further investigations are expected to indicate whether there is a connection between the src family proteins and HA/RHAMM.

In summary, we have shown that HA/RHAMM interactions result in rapid tyrosine phosphorylation that leads to locomotion in *ras* transformed fibroblasts. We propose that a target of this signaling pathway is focal adhesions and the focal

adhesion kinase, FAK. These results further identify, for the first time, hyaluronan as an important regulator of focal adhesion turnover.

Acknowledgements

This work was supported by the National Institutes of Health Grant CA 51540, a CHRF Scholarship to E.T. and a MRC scholarship to C.L.H.

REFERENCES

Abercrombie, M. 1980. The crawling movement of metazoan cells. *Proc. R. Soc. Lond. B.* 207:614-628.

Abercrombie, M., G.A. Dunn, and J.P. Heath. 1977. The shape and movement of fibroblasts in culture. *In* Cell and Tissue Interactions. J.W. Lash, and M.M. Burger, editors. Raven Press, New York. 57-90.

Abercrombie, M., J.E.M. Heaysman, and S.M. Pegrum. 1970. The locomotion of fibroblasts in culture. I. Movements of the leading edge. *Exp. Cell Res.* 59:393-398.

Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, and Y. Fukami. 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 262:5592-5595.

Aznavoorian, S.A., M.L. Strache, H. Krutzsch, E. Schiffman, and L.A. Liotta. 1990. Signal transduction for chemotaxis and haptotaxis by matrix molecules in tumor cells. *J. Cell Biol.* 110:1427-1438.

Banerjee, S.D. and B.P. Toole. 1992. Hyaluronan-binding protein in endothelial cell morphogenesis. *J. Cell Biol.* 119:643-652.

Beckerle, M.C. 1990. The adhesion plaque protein, talin, is phosphorylated *in vivo* in chicken embryo fibroblasts exposed to a tumor-promoting phorbol ester. *Cell Reg.* 1:227-236.

Beckerle, M.C., K. Burridge, G.N. DeMartino, and D.E. Croall. 1987. Colocalization of calcium dependant protease II and one of its substrates at sites of cell adhesion. *Cell.* 51:569-577.

Bell, L., D.J. Luthringer, J.A. Madri, and S.L. Warren. 1992. Autocrine angiotensin system regulation of bovine aortic endothelial cell migration and plasminogen activator involves modulation of proto-oncogene $pp60^{c\text{-}src}$ expression. *J. Clin Invest.* 89:315-320.

Bernanke, D.H., and R.R. Markwald. 1979. Effects of hyaluronic acid on cardiac cushion tissue cells in collagen matrix cultures. *Texas Rep. Biol. Med.* 39:271-285.

Bolen, J.B., P.A. Thompson, E. Eiseman, and I.D. Horak. 1991. Expression and interactions of the src family of tyrosine kinases in T lymphocytes. *Adv. Cancer Res.* 57:103-149.

Bottaro, D.P., J.S. Rubin, D.L. Faletto, A.M. Chan, T.E. Kmiecik, G.F. Vande Woude, and S.A. Aaronson. 1991. Identification of the hepatocyte growth factor receptor as the *c-met* proto-oncogene product. *Science (Wash. DC).* 251: 802-804.

Boudreau, N., E. A. Turley, and M. Rabinovitch. 1991. Fibronectin, hyaluronan and hyaluronan binding protein contribute to increased ductus arteriosus smooth muscle cell migration. *Devel. Biol.* 143:235-247.

Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487-525.

Burridge, K., C.E. Turner and L.H. Romer. 1992. Tyrosine phosphorylation of $pp125^{FAK}$ accompanies cell adhesion to the extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.* 119:893-903.

Chiguet-Ehrismann, R., P. Kalla, C.A. Pearson, K. Beck and M. Chiguet. 1988. Tenascin interferes with fibronectin action. *Cell* 53:383-390.

Comoglio, P.M., M.F. DiRenzo, G. Tarone, F.G. Giancotti, L. Naldini, and P.C. Marchisio. 1984. Detection of phosphotyrosine-containing proteins in the detergent-insoluable fraction of RSV-transformed fibroblasts by azobenzene phosphate antibodies. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:483-489.

Cooper, J.A., D.J. Loftus, C. Freiden, J. Bryan, and E.L. Elson. 1988. Localization and mobility of gelsolin in cells. *J. Cell Biol.* 106:1226-1240.

Copp, A.J., and M. Bernfield. 1988a. Glycosaminoglycans vary in accumulation along the neuraxis during spinal neurulation in the mouse embryo. *Dev. Biol.* 130:573-582.

Copp, A.J., and M. Bernfield. 1988b. Accumulation of basement membrane associated hyaluronate is reduced in the posterior neuropore region of mutant (curly tail) mouse embryos developing spinal neural tube defects. *Dev. Biol.* 130:583-590.

Davis, S., M.L. Lu, J.A. Butler, B.J. Druker, T.M. Roberts, Q. An, and L.B. Chen. 1991. Presence of an SH2 domain in the actin-binding protein tensin. *Science (Wash. DC.).* 252:712-715

DeClue, J.E., and G.S. Martin. 1987. Phosphorylation of talin at tyrosine in Rous sarcoma virus-transformed cells. *Mol. Cell. Biol.* 7:941-955.

Egan, S.E., G.A. McClarty, L. Jarolim, J.A. Wright, I. Spiro, G. Hager, and A.H. Greenberg. 1987. Expression of H-*ras* correlates with metastatic potential: evidence of direct regulation of the metastatic phenotype in 10T1/2 and NIH 3T3 cells. *Mol. Cell. Biol.* 7:830-837.

Ferrel,J.E., and G.S. Martin. 1989. Tyrosine-specific phosphorylation is regulated by glycoprotein IIb, IIIa in platelets. *Proc. Natl. Acad. Sci. USA.* 86:2234-2238.

Freed, E., J. Gailet, P. van der Geer, E. Ruoslahti, and T. Hunter. 1989. A novel integrin β integrin subunit is associated with the vitronectin receptor subunit (α_v) in a human osteosarcoma cell line and is a subunit for protein kinase C. *EMBO (Eur. Mol. Biol. Org.) J.* 8:2955-2965.

Gingell, D., and N. Owens. 1992. How do cells sense and respond to adhesive contacts? Diffusion-trapping of laterally mobile membrane proteins of maturing adhesions may initiate signals leading to local assembly response and lamella formation. *J. Cell Sci.* 101:255-266.

Gaudry, M., A.C. Caon, C. Gilbert, S. Lille, and P.H. Caccache. 1992. Evidence for the involvement of tyrosine kinases in the locomotory responses of human neutrophils. *J. Leukoc. Biol.* 51:103-108.

Glenney, J.R., and L. Zokas. 1989. Novel tyrosine kinase substrates from Rous sarcoma virus transformed cells are present in the membrane skeleton. *J. Cell Biol.* 108:2401-2408.

Goldman, R.D., J.A. Schloss, and J.M. Starger. 1976. Organizational changes of actinlike microfilaments during animal cell movement. *In* Cell Motility. A. Muscle and Non-Muscle Cells. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York. 217-245.

Guan, J.-L., and D. Shalloway. 1992. Regulation of focal adhesions-associated

protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature (Lond.)*. 358:690-692.

Guan, J.-L., J.A. Trevithick, and R.O. Hynes. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Reg.* 2:951-964.

Hadden, C.M., and J.H. Lewis. 1991. Hyaluronan as a propellent for epithelial movement: the development of semicircular canals in the inner ear of Xenopus. *Development*. 112:541-550.

Hakansson, L., R. Hallgren, and P. Venge. 1980a. Regulation of granulocyte function by hyaluronic acid. *J. Clin. Invest.* 66:298-305.

Hakansson, L., R. Hallgren, P. Venge, G. Artursson, and S. Vedung. 1980b. Hyaluronic acid stimulates neutrophil function *in vitro* and *in vivo*. *Scand. J. Infect. Dis. Suppl.* 24:54-56.

Hamaguchi, M., and H. Hanafusa. 1987. Association of $p60^{src}$ with triton X-100-resistant cellular structure correlates with morphological transformation. *Proc. Natl. Acad. Sci. USA*, 84:2312-2316.

Hanks, S.K., M.B. Calalb, M.C. Harper and S.K. Patel. 1992. Focal adhesion protein tyrosine kinase phosphorylated in response to cell spreading on fibronectin. *Proc. Natl. Acad. Sci. USA*, 89:8487-8491.

Hardwick, C., K. Hoare, R. Owens, H.P. Hohn, M. Hook, D. Moore, V. Cripps, L. Austen, D.M. Nance and E.A. Turley. 1992. Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. *J. Cell Biol.*, 117:1343-1350.

Harris, A.K. 1973. Cell surface movements related to cell locomotion. In Locomotion of Tissue Cells. *Ciba Fdn. Symp.*, 14:3-27.

Hart, I.R., M. Birch and J.F. Marshall. 1991. Cell adhesion receptor expression during melanoma progression and metastasis. *Cancer Metastasis Rev.*, 10:115-128.

Hirst, R., A. Horwitz, C. Buck and L. Rohrschneider. 1986. Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natl. Acad. Sci. USA*, 83:6470-6474.

Huzar, G., M. Willets and M. Corrales. 1990. Hyaluronic acid (sperm select) improves retention of sperm motility and velocity in normospermic and oligospermic specimens. *Fert. Steril.*, 54:1127-1134.

Iozzo, R.V. 1985. Proteoglycans: structure, function and the role in neoplasia. *Lab. Invest.*, 53:373-396.

Izzard, C.S., and Lochner, L.R. 1980. Formation of cell-to-substrate contacts during fibroblast motility: an interference-reflexion study. *J. Cell Sci.* 42:81-116

Izumi, T., Y. Saeki, Y. Akamuru, F. Takaku, and M. Kasuga. 1988. Requirement for receptor intrinsic tyrosine kinase activities during ligand-induced membrane ruffling of KB cells: essential sites of src-related growth factor receptor kinases. *J. Biol. Chem.* 263:10386-10393.

Jaken,S., K. Leach, and T. Klauck. 1989. Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. *J. Cell Biol.* 109:697-704.

Juliano, R.L. and S. Haskell. 1993. Signal transduction from the extracellular matrix. *J. Cell Biol.* 120:577-585.

Kanner, S.B., A.B. Reynolds, and J.T. Parsons. 1991a. Tyrosine phosphorylation of a 120-kilodalton pp60^{src} substrate upon epidermal growth factor and platelet-derived growth factor receptor stimulation and in Polyoma virus middle-T-antigen-transformed cells. *Mol. Cell. Biol.* 11:713-720.

Kanner, S.B., A.B. Reynolds, R.R. Vines, and J.T. Parsons. 1990. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 87:3328-3332.

Kanner, S.B., A.B. Reynolds, H.-C.R. Wang, R.R. Vines, and J.T. Parsons. 1991b. The SH2 and SH3 domains of pp60^{src} direct stable association with tyrosine phosphorylated proteins p130 and p110. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1689-1698.

Komada, N., and N. Kitamura. 1993. The cell dissociation and motility triggered by scatter factor/hepatocyte growth factor are mediated through the cytoplasmic domain of the c-Met receptor. *Oncogene* 8:2381-2390.

Kornberg, L., H.S. Earp, J.T. Parsons, M. Schaller, and R.L. Juliano. 1992. Cell adhesion and integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* 267:23439-23442.

Kornberg, L.J., H.S. Earp, C.E. Turner, C. Prockop, and R.L. Juliano. 1991. Signal transduction by integrins: Increased protein tyrosine phosphorylation caused by clustering of β_1 integrins. *Proc. Natl. Acad, Sci. USA.* 88:8392-8396.

Kornovski, B.J., J. McCoshen, J. Kredentser and E.A. Turley. 1993. The regulation of sperm motility by a novel hyaluronan receptor. *Fert. Steril.*, in press.

Lester, B.R., and J.B. McCarthy. 1992. Tumor cell adhesion to the extracellular matrix and signal transduction mechanisms implicated in tumor cell motility, invasion and metastasis. *Cancer Metastasis Rev.* 11:31-44.

Lightner, V.A. and H.P. Erickson. 1990. Bending to hexabrachion (tenascin) to the extracellular matrix and substratum and its effect on cell adhesion. *J. Cell Sci.* 95:263-277.

Lipfert, L. B. Haimovich, M.D. Schaller, B.S. Cobb, J.T. Parsons, and J.S. Brugge. 1992. Integrin-dependent phosphorylation and activation of the protein tyrosine kinase $pp125^{FAK}$ in platelets. *J. Cell Biol.* 119:905-912.

Litchfield, D.W., and E.H. Ball. 1986. Phosphorylation of the cytoskeletal protein talin by protein kinase C. *Biochem. Biophys. Res. Commun.* 134:1276-1283.

Lo, S.H., and L.B. Chen. 1994. Focal adhesion as a signal transduction organelle. *Cancer Metastasis Rev.* 13:9-24.

Maher, P.A., E.B. Pasquale, J.Y.J. Wang, and S.J. Singer. 1985. Phosphotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells. *Proc. Natl. Acad. Sci. USA.* 82:6576-6580.

Matten, W.T., M. Aubry, J. West, and P.F. Manness. 1990. Tubulin is phosphorylated on tyrosine by $pp60^{c\text{-}src}$ in nerve growth cone membranes. *J. Cell Biol.* 111:1959-1970.

McCarthy, J.B., M.L. Basara, S.L. Palm, D.F. Sas, and L.T. Furcht. 1985. The role of cell adhesion proteins laminin and fibronectin in the movement of malignant and metastatic cells. *Cancer Metastasis Rev.* 4:125-152.

McCarthy, J.B., S.J. Hager, and L.T.F. Furcht. 1986. Human fibronectin contains distinct adhesion- and motility-promoting domains for metastatic melanoma cells. *J. Cell Biol.* 102:179-188.

Mensing, H., A. Albini, T. Kreig, B.F. Pontz, and P.K. Muller. 1985. Enhanced chemotaxis of tumor-derived and virus-transformed cells to fibronectin and fibroblast conditioned medium. *Int. J. Cancer.* 33:43-48.

Mueller, S.C., Y. Yeh, and W.-T. Chen. 1992. Tyrosine phosphorylation of membrane proteins mediates cellular invasion by transformed cells. *J. Cell Biol.*

119:1309-1325.

Murphy-Ullrich, J.E., V.A. Lightner, I. Anthil, Y.Z. Yan, H.P. Erickson and M. Hook. 1991. Focal adhesion integrity is down regulated by alternatively spliced domains of human tenascin. *J. Cell Biol.*, 115:1127-1136.

Nakamura, S.I., and H. Yamamura. 1989. Thrombin and collagen induce rapid phosphorylation of a common set of cellular proteins on tyrosine in human platelets. *J. Biol. Chem.* 264:7089-7091.

Nigg, E.A., B.M. Sefton, T. Hunter, G. Walter, and S.J. Singer. 1982. Immunofluorescence localization of the transforming protein of Rous sarcoma virus with antibodies against a synthetic src peptide. *Proc. Natl. Acad. Sci. USA.* 79:5322-5326.

Pasquale, E.B., P.A. Maher, and S.J. Singer. 1986. Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 83:5507-5511.

Pauli, B.V., D.E. Schwartz, E.J. Thonar, and K.E. Kuetttner. 1983. Tumor invasion and host extracellular matrix. *Cancer Metastasis Rev.* 2:129-152.

Powell, M.A., and J.R. Glenney. 1987. Regulation of calpactin I phospholipid binding by calpactin light-chain binding and phosphorylation by $p60^{v\text{-}src}$. *Biochem. J.* 247:321-328.

Reynolds, A.B., L. Herbert, J.L. Cleveland, S.T. Berg, and J.R. Gaut. 1992. p120, a novel substrate of protein tyrosine kinase receptors and of $p60^{v\text{-}src}$ is related to cadherin-binding factor B-catenen, plakoglobin and *armadillo*. *Oncogene*. 7:2439-2445.

Rinnerthaler, G., B. Geiger, and J.V. Small. 1988. Contact formation during fibroblast locomotion: involvement of membrane ruffles and microtubules. *J. Cell Biol.* 106:747-760.

Rohrschneider, L.R. 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product. *Proc. Natl. Acad. Sci. USA.* 77:3514-3518.

Rorhschneider, L.R., and L.E. Gentry. 1984. Subcellular locations of retroviral transforming proteins define multiple mechanisms of transformation. *Adv. Viral Oncol.* 4:269-306.

Rorhschneider, L.R. and L.M. Najita. 1984. Detection of the v-abl gene product at

cell-substratum contact sites in Ableson murine leukemia virus-transformed fibroblasts. *J. Virol.* 51:547-552.

Rohrschneider, L.R., M. Rosok, and K. Shriver. 1982. Mechanism of transformation by Rous sarcoma virus: events within adhesion plaques. *Cold Spring Harbor Symp. Quant. Biol.* 46:956-965.

Rothberg, K.C., J.E. Henser, W.C. Donzell, Y.-S. Ying, J.R. Glenney, and R.G.W. Anderson. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell.* 68:673-687.

Ruoslahti, E. 1992. Control of cell motility and tumor invasion by extracellular matrix interactions. *Br. J. Cancer.* 66:239-242.

Sage, E.H. and P. Bornstein. 1991. Extracellular proteins that modulate cell-matrix interactions. *J. Biol. Chem.* 266:14831-14834.

Samuel, S.K., R.A.R. Hurta, M.A. Spearman, J.A. Wright, E.A. Turley, and A.H. Greenberg. 1993. TGF-β_1 stimulation of cell locomotion utilizes the hyaluronan receptor RHAMM and hyaluronan. *J. Cell Biol.*, 123:749-758.

Schaller, M.D., C.A. Borgman, B.S. Cobb, R.R. Vines, A.B. Reynolds, and J.T. Parsons. 1992. pp125^{FAK}, a structurally unique protein tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* 89:5192-5196.

Schliwa, M., T. Nakamura, K.R. Porter, and V. Euteneur. 1984. A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. *J. Cell Biol.* 99:1045-1089.

Schor, S.L., A.M. Schor, A.M. Grey, J. Chen, G. Rushton, N.E. Grant, and I. Eins. 1989. Mechanisms of action of the migration stimulating factor produced by fetal and cancer patient fibroblasts: effect on hyaluronic acid synthesis. *In vitro Cell Devel. Biol.* 25:737-745.

Sefton, B.M., T. Hunter, E.H. Ball, and S.J. Singer. 1981. Vinculin: a cytoskeletal target of the transforming protein of Rous Sarcoma virus. *Cell.* 24:165-174.

Segall, J.E., and G. Gerisch. 1989. Genetic approaches to cytoskeletal function and control of cell motility. *Curr. Opin. Cell Biol.* 1:44-50.

Shaw, L.M., J.M. Messier, and A.M. Mercurio. 1990. The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the $\alpha_6\beta_1$ integrin. *J. Cell Biol.* 110:2167-2174.

Shriver, K., and L.R. Rohrschneider. 1981a. Spatial and enzymatic interaction of $pp60^{v\text{-}src}$ with cytoskeletal proteins in isolated adhesion plaques and junctions from RSV-transformed NRK cells. *Cold Spring Harbor Conf. Cell Proliferation.* 8:1247-1262.

Shriver, K., and L.R. Rohrschneider. 1981b. Organization of $pp60^{v\text{-}src}$ and selected cytoskeletal proteins with adhesion plaques and junctions of Rous sarcoma virus transformed rat cells. *J. Cell. Biol.* 89:525-535.

Stamenkovic, I., A. Aruffo, M. Amiot, and B. Seed. 1991. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronan bearing cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:343-348.

Starkey, J.R. 1990. Cell-matrix interactions during tumor invasion. *Cancer Metastasis Rev.* 9:113-123.

Takahashi, K., U.I. Heine, J.L. Junker, N.H. Colburn, and J.M. Rice. 1986. Role of cytoskeletal changes and expression of the H-*ras* oncogene during promotion of neoplastic transformation in mouse epidermal JB6 cell. *Cancer Res.* 46:5923-5932.

Tapley, P., A. Horwitz, C. Buck, K. Duggan, and L. Rohrschneider. 1989. Integrins isolated from Rous sarcoma virus-transformed chicken embryo fibroblasts. *Oncogene.* 4:325-333.

Thomas, L., H.R. Byers, J. Vink, and I. Stamenkovic. 1992. CD44H regulates tumor cell migration on hyaluronate-coated substrate. *J. Cell Biol.* 118:971-977.

Toole, B.P. 1982. Developmental role of hyaluronate. *Connect. Tissue Res.* 10:93-100.

Toole, B.P., R.L. Goldberg, G. Chi-Rosso, C.B. Underhill, and R. Orkin. 1984. Hyaluronate-cell interactions. *In* The Role of Extracellular Matrix in Development. R. L. Trelstad, editor. Alan R. Liss Inc., New York. 43-66.

Toole, B.P., S.F. Munaim, S. Welles, and C.B. Knudson. 1989. Hyaluronate-cell interactions and growth factor regulation of hyaluronate synthesis during limb development. Biology of Hyaluronan. *Ciba Found. Symp.* 143:150-169.

Turley, E.A. 1984. Proteoglycans and cell adhesion: their putative role during tumorigenesis. *Cancer Metastasis Rev.* 3:325-339.

Turley, E.A. 1989a. The role of a cell-associated hyaluronan-binding protein in fibroblast behaviour. *In* Biology of Hyaluronan. *Ciba Found. Symp.* 143: 121-137.

Turley, E.A. 1989b. Hyaluronic acid stimulates protein kinase activity in intact cells and in an isolated protein complex. *J. Biol. Chem.* 264:8951-8955.

Turley, E.A. 1992. Hyaluronan and cell locomotion. *Cancer Metastasis Rev.* 11:21-30.

Turley, E.A., L. Austen, K. Vandeligt and C. Clary. 1991. Hyaluronan and a cell-associated hyaluronan binding protein regulate the locomotion of *ras*-transformed cells. *J. Cell Biol.* 112:1041-1047.

Turley, E.A., P. Brassel, and D. Moore. 1990. A hyaluronan-binding protein shows a partial and temporally regulated codistribution with actin on locomoting chick heart fibroblasts. *Exp. Cell Res.* 187:243-249.

Turner, C.E., J.R. Glenney, and K. Burridge. 1990. Paxillin: a new vinculin binding protein present in focal adhesions. *J. Cell Biol.* 111:1059-1068.

Turner, C.E., F.M. Pavalko, and K. Burridge. 1989. The role of phosphorylation and limited proteolytic cleavage of talin and vinculin in the disruption of focal adhesion integrity. *J. Biol. Chem.* 264:11938-11944.

Uehara, Y., Y. Murakami, Y. Sugimoto, and S. Mizuno. 1989. Mechanism of reversion of Rous sarcoma virus transformation by herbimycin A: reduction of total phosphotyrosine levels due to reduced kinase activity and increased turnover of $p60^{v\text{-}src}$. *Cancer Res.* 49:780-785.

Volberg, T., B. Geiger, R. Dror, and Y. Zick. 1991. Modulation of intercellular adherens-type junctions and tyrosine phosphorylation of their components in RSV-transformed cultured chick lens cells. *Cell Regul.* 2:105-120.

Weidner, K.M., M. Sachs, and W. Brichmeier. 1993. The Met receptor tyrosine kinase transduces motility, proliferation and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. *J. Cell Biol.* 121:145-154.

Weigel, P.H., S.J. Frost, R.D. LeBoeuf, and C.T. McGary. 1989. The specific interaction between fibrinogen and hyaluronan: possible consequences in haemostasis, inflammation and wound healing. Biology of Hyaluronan. *Ciba Found. Symp.* 143:248-264.

Weigel, P.H., G.M. Fuller, and R.D. LeBoeuf. 1986. A model for the role of hyaluronic and fibrinogen in the early events during the inflammatory response and wound healing. *J. Theor. Biol.* 119:219-234.

Werth, D.K., J.E. Niedel, and I. Pastan. 1983. Vinculin, a cytoskeletal substrate for protein kinase C. *J. Biol. Chem.* 258:11423-11426.

Werth, D.K., and I. Pastan. 1984. Vinculin phosphorylation in response to calcium and phorbol esters in intact cells. *J. Biol. Chem.* 259:5264-5270.

West, D.C., and S. Kumar. 1989. Hyaluronan and angiogenesis. Biology of Hyaluronan. *Ciba Found. Symp.* 143:157-207.

Woods, A., and J.R. Couchman. 1992. Protein kinase C involvement in focal adhesion formation. *J. Cell Sci.* 101:277-290.

Wu, H., and J.T. Parsons. 1993. Cortactin, an 80/85-kilodalton $pp60^{src}$ substrate, is a filamentous actin binding protein enriched in the cell cortex. *J. Cell Biol.* 120:1417-1426.

Yusa, T., C. M. Blood, and B. R. Zetter. 1989. Tumor cell interactions with elastin: implications for pulmonary metastasis. *Am. Rev. Respir. Dis.* 140:1458-1462

Zachary,I., and E. Rozengurt. 1992. Focal adhesion kinase ($p125^{FAK}$): a point of convergence in the action of neuropeptide, integrins and oncogenes. *Cell.* 71:891-894.

Zigmund, S.H. 1989. Cell locomotion and chemotaxis. *Curr. Opin. Cell Biol.* 1:80-86.

Figure 1. Locomotion of C3 cells with (○) or without (□) the addition of HA (10 ng/ml). C3 cells, 24 hours after subculture, were analyzed by computer timelapse image analysis for 10 hours in the presence (○) or absence (□) of HA. Within 2 hours of HA addition, cells were locomoting maximally, after which locomotion decreased slightly. Values remain elevated over the controls, for the remaining 8 hours. Values represent the mean ± SEM. $n = 90$ cells.

Figure 1

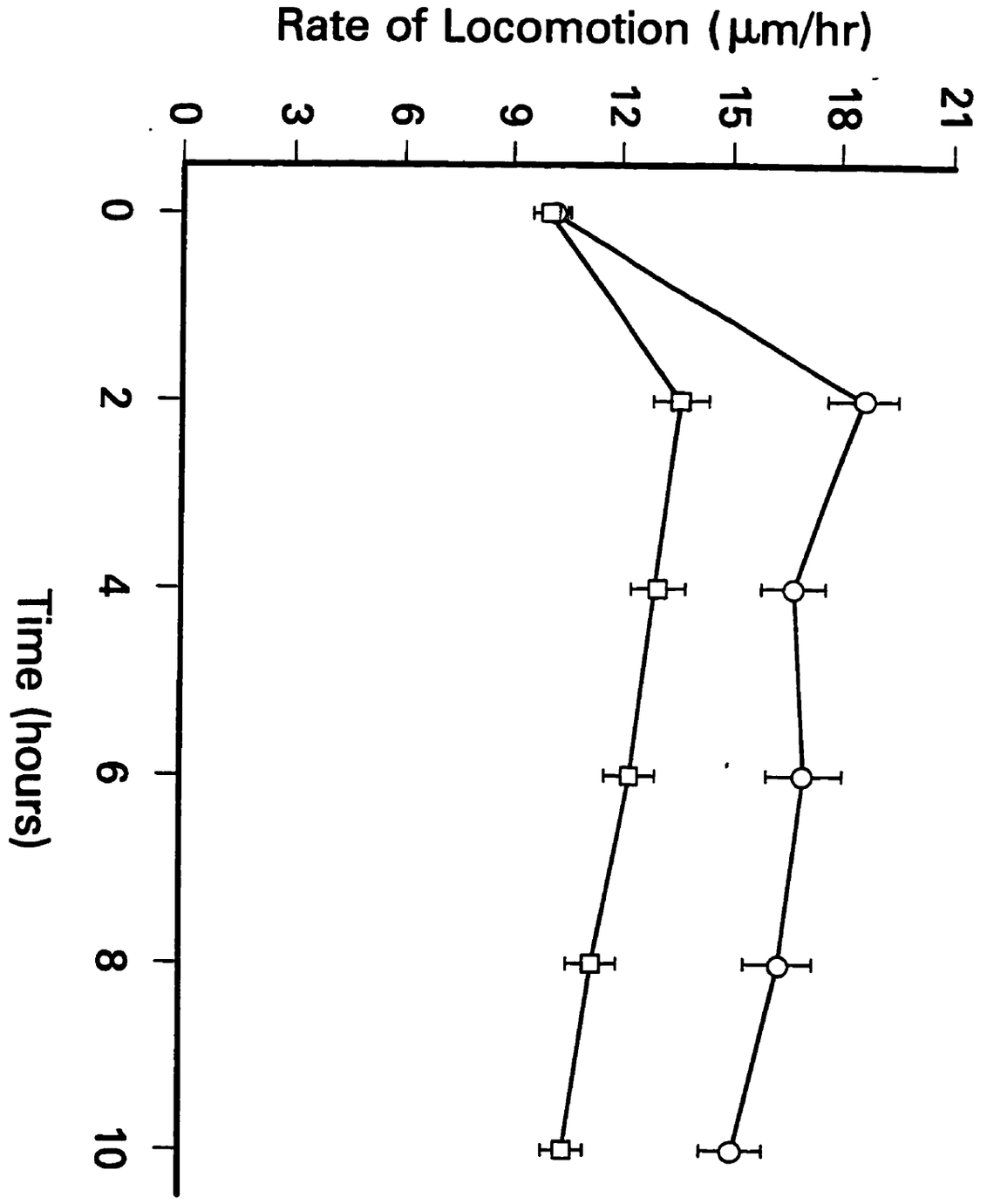


Figure 2. Effect of HA addition on tyrosine phosphorylation. C3 cells were incubated in the absence (lane 1) or presence (lanes 2-5) of 10 ng/ml of HA for 20 s (lane 2), 1 min (lane 3), 5 min (lane 4), or 10 min (lane 5) prior to cell lysis with RIPA buffer. Equal protein concentrations from the lysates were electrophoresed on SDS-PAGE gels and either stained with Coomassie Blue (*C*) or transferred to nitrocellulose, immunoblotted using an antiphosphotyrosine MAb (4G10, UBI) and developed with ECL (*A*). Densitometric analysis (*B*) reveals increased phosphotyrosine in bands at 125, 115, 75, 60, 56 and 50. Densitometry of 3 bands (b1, b2 and b3) from the Coomassie stain is shown (*D*). The molecular weight markers (M) are marked at 190, 125, 88, 65, 56, 38 and 33.5 kDa.

Figure 2

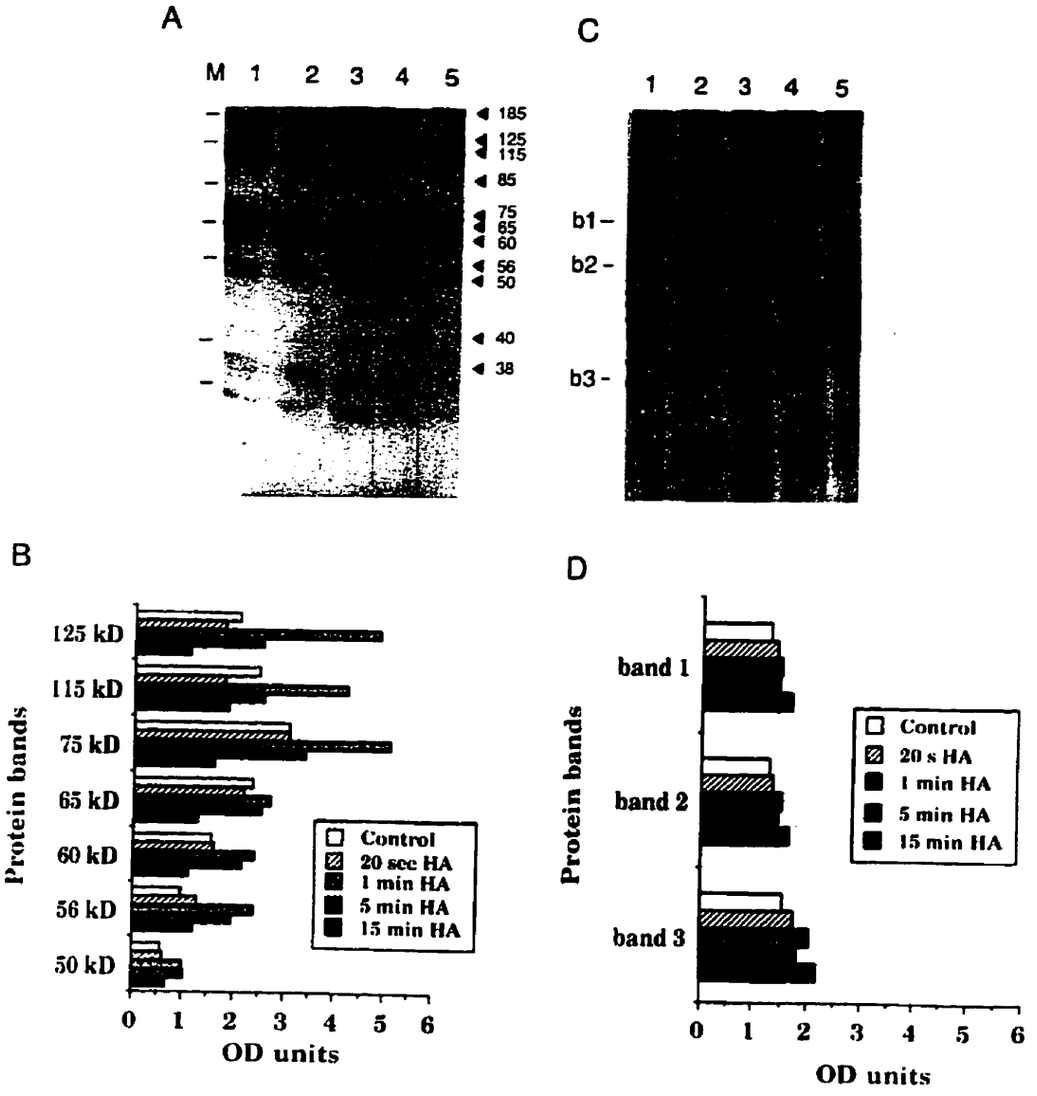

A
M 1 2 3 4 5
185
125
115
85
75
65
60
56
50
40
38
C
1 2 3 4 5
b1
b2
b3
B
125 kD
115 kD
75 kD
65 kD
60 kD
56 kD
50 kD
Protein bands
Control
20 sec HA
1 min HA
5 min HA
15 min HA
0 1 2 3 4 5 6
OD units
D
band 1
band 2
band 3
Protein bands
Control
20 s HA
1 min HA
5 min HA
15 min HA
0 1 2 3 4 5 6
OD units

Figure 3. Immunofluorescence localization of phosphotyrosine in HA stimulated cells. C3 fibroblasts were incubated in the absence (*a-c*) or presence of 10 ng/ml HA for 1 min (*d-f*), or 15 min (*g,h*) prior to fixation and staining with anti-phosphotyrosine. Cells exposed to HA for 1 min show an increased staining for phosphotyrosine that drops below control levels by 15 min. Mouse IgG control is shown (*i*). Size bar represents 25 μm.

Figure 3

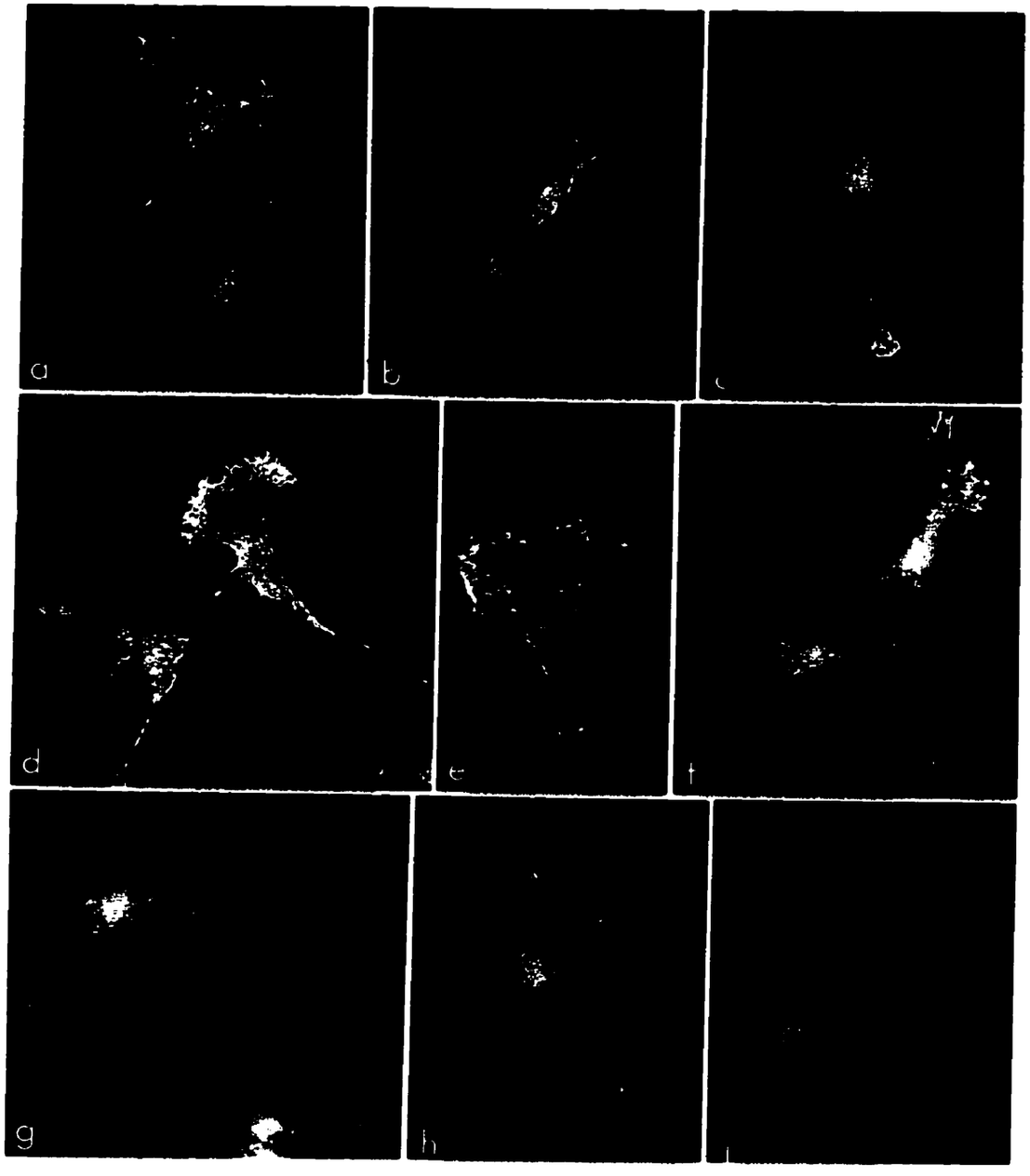


Figure 4. Anti-RHAMM mimics HA responses. *A*. Anti-RHAMM (lane *2*) or control pre-immune (lane *1*) serum was added to C3 fibroblasts and the motility recorded for 2 h. Anti-RHAMM, like HA, addition results in a significant increase ($p < 0.001$) in cell locomotion. *B*. Anti-RHAMM (lane *2*) or pre-immune (lane *1*) serum was added to C3 cells for 1 min, followed by cell lysis, SDS-PAGE and anti-phosphotyrosine immunoblotting. Anti-RHAMM, like HA, stimulated an increase in tyrosine phosphorylation of several protein bands. Markers (*M*) are marked at 211, 119, 98, 80.6, and 64.4. *C*. C3 cells were treated with pre-immune serum (*a-c*) for 1 min, or anti-RHAMM for 1 min (*d-f*), or 15 min (*g,h*) prior to fixation and indirect immunofluorescent staining with anti-phophotyrosine MAb. Like HA treatment, anti-RHAMM treatment results in a transient increase in phosphotyrosine staining that drops below control levels by 15 min after stimulation. Mouse IgG control shown (*i*). Size bar represents 25 μm.

Figure 4 A & B

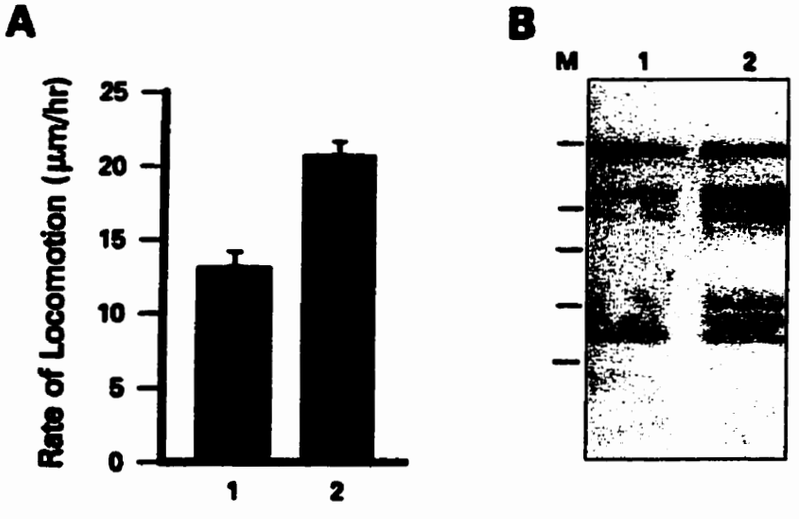


Figure 4C

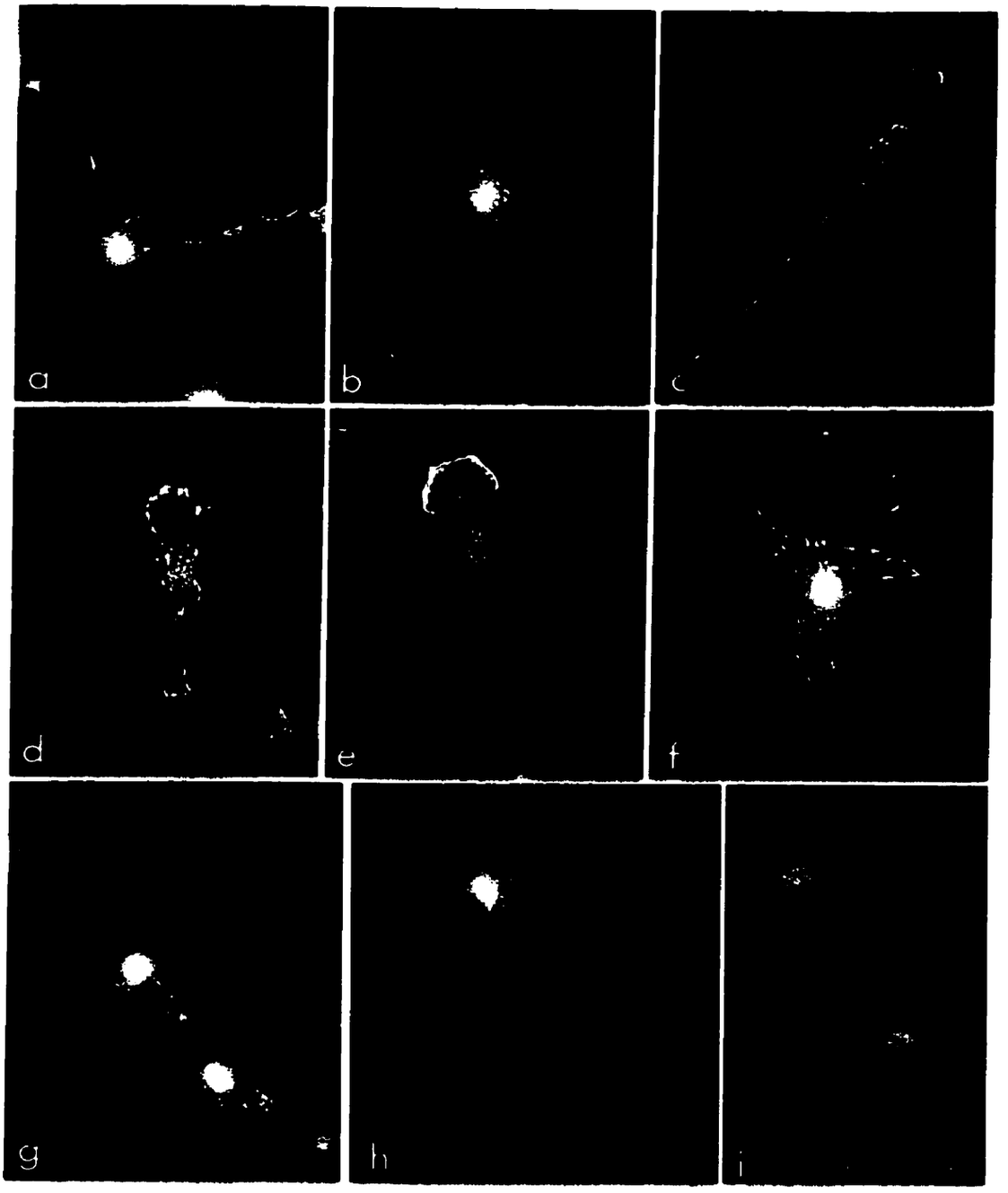


Figure 5. Protein tyrosine kinase inhibitors diminish HA-mediated tyrosine phosphorylation as detected by anti-phosphotyrosine immunoblotting. *A*. C3 fibroblasts were incubated with genistein (10 μg/ml; lanes 3 and 4) or DMSO for controls (lanes 1 and 2) for 10 min prior to incubation with (lanes 2 and 4) or without (lanes 1 and 3) HA (10 ng/ml) for 1 min. Lysates were subjected to SDS-PAGE and anti-phosphotyrosine immunoblot analysis. Genistein addition diminishes the HA stimulated phosphorylation of the bands at 125 and 115 kDa, as seen by densitometric analysis (*B*). The molecular weight markers (M) are marked at 211, 119, 98, 80.6 and 64.4 kDa.

Figure 5

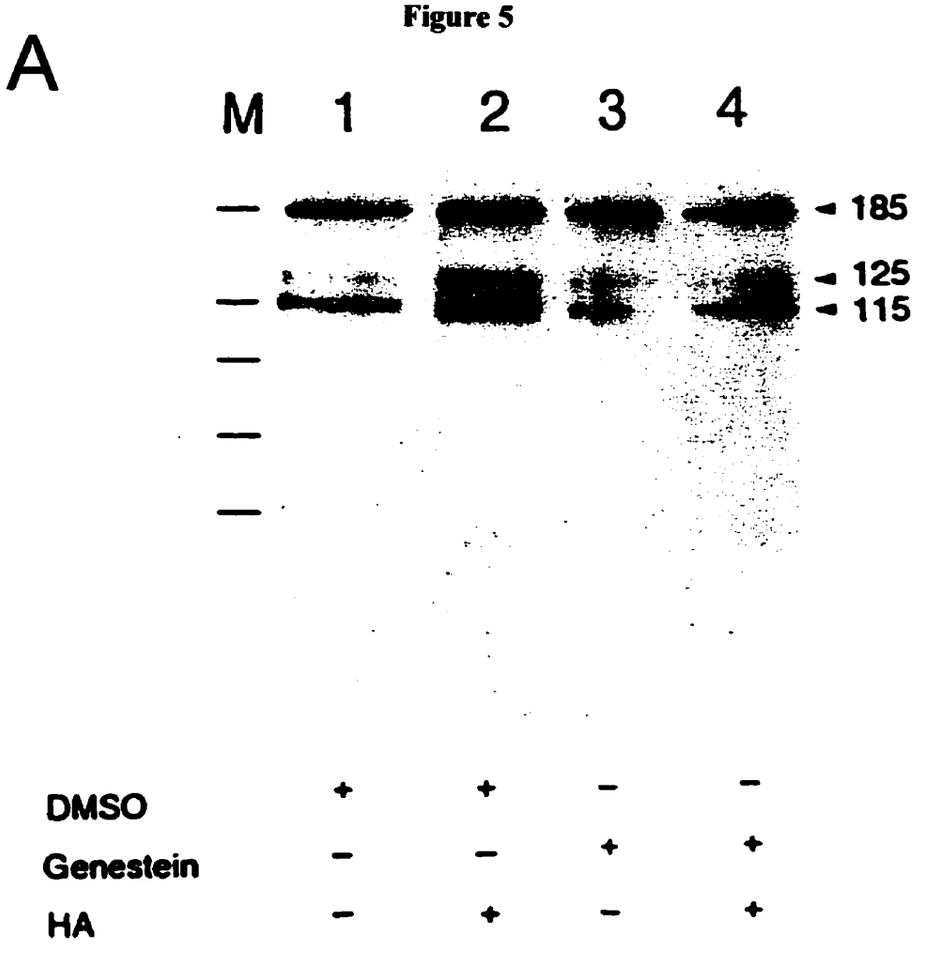

A
M
1
2
3
4
185
125
115
DMSO
Genestein
HA
+
+
−
−
−
−
+
+
−
+
−
+
B
Protein bands
p125
p115
0.0
0.2
0.4
0.6
0.8
1.0
1.2
OD units
DMSO
DMSO + HA
Genistein
Genistein + HA

Figure 6. Genistein inhibits HA mediated locomotion. C3 cells, 24 hours after subculturing, were treated with genistein (10 μg/ml; □, ●) or control treatment (○), and then the motility was recorded with (○, ●) or without (□) HA (10 ng/ml) addition. The genistein + HA (●) treated cells locomote basally, in contrast to the control treatment + HA (○) cells. Values represent the mean ± SEM. $n = 90$ cells.

Figure 6

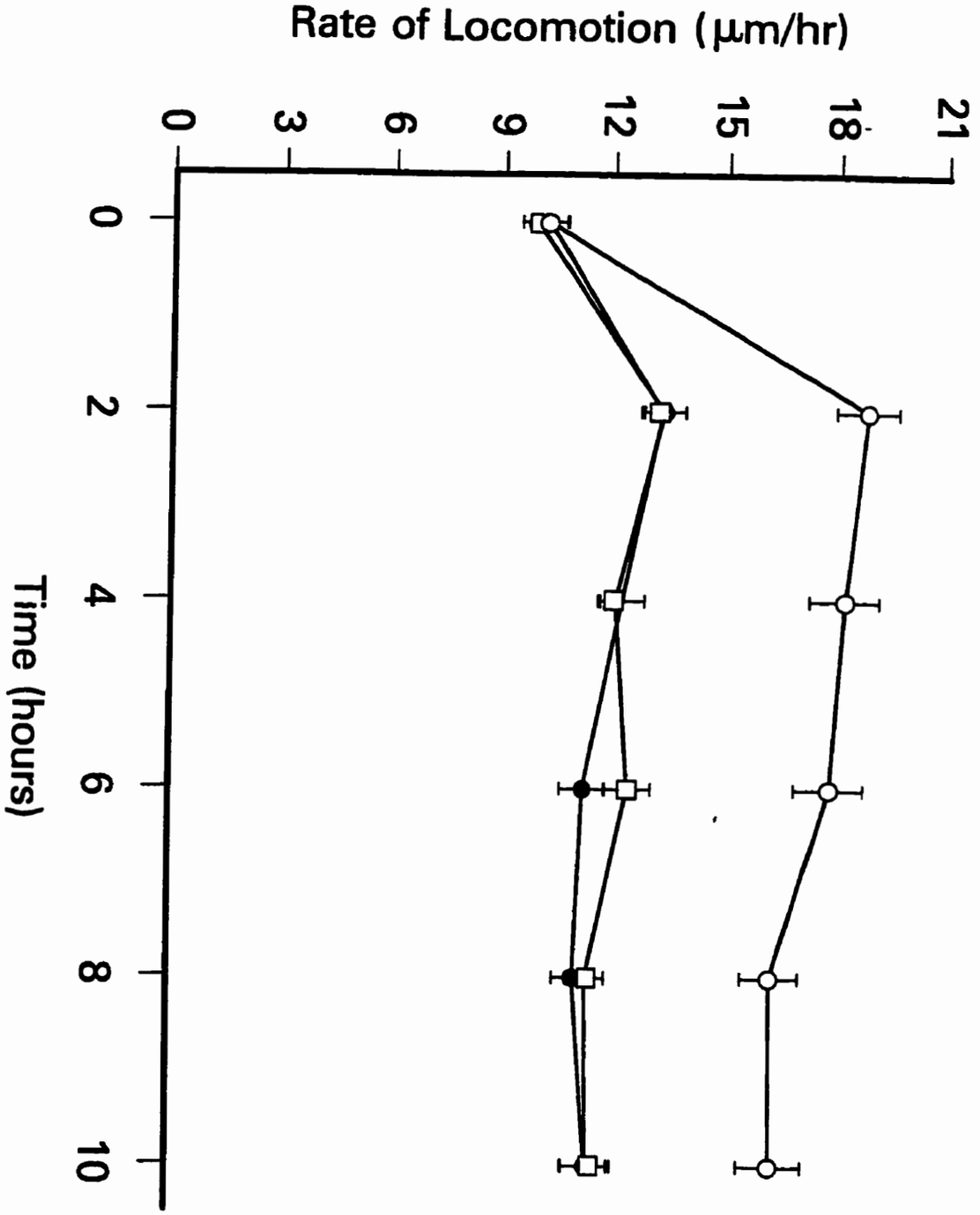


Figure 7. Herbimycin A inhibits HA promoted motility. C3 cells, 24 hours after subculturing, were treated with control treatment (*A*) or Herbimycin A (0.5 μg/ml; *B*) for 4 hours prior to HA (10 ng/ml) addition. The cell motility was recorded for 2 hours using computer timelapse image analysis and the motility rates were grouped accordingly (A: 0 - 2.0, B: 2.1 - 4.0, C: 4.1 - 6.0, D: 6.1 - 8.0, E: 8.1-10.0, F: $\geq$ 10.1 μm/min). It is apparent that a large population the cells pretreated with herbimycin A (*B*) have a lower motility rate than the HA treated controls (*A*). $n = 90$.

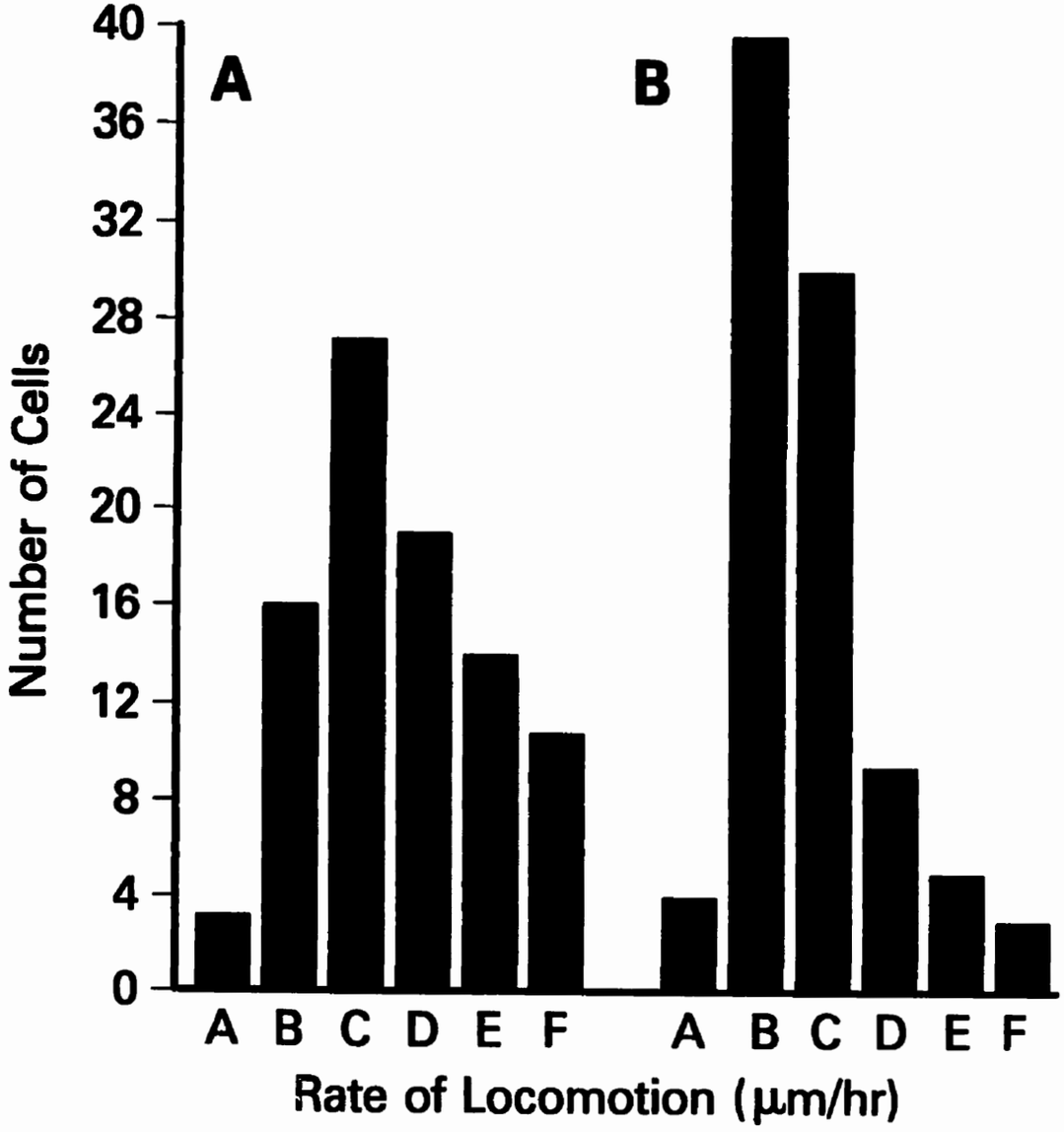

40
36
32
28
24
20
16
12
8
4
0
A
B
Number of Cells
A B C D E F
A B C D E F
Rate of Locomotion (µm/hr)

Figure 7

Figure 8. The effect of genistein added after HA stimulation. C3 cell cultures were treated with 10 ng/ml HA (○, □, ●) before or after genistein (10 μg/ml) addition. The sample treated with HA only (○) illustrates a typical HA mediated locomotory response. Genistein added 10 min prior to HA completely inhibits the HA promoted locomotion (●), but genistein addition 10 min after HA addition does not affect the sharp increase in locomotion (□). However the post-HA genistein treated cells do not have a high maintained rate of locomotion. Values represent the mean $\pm$ SEM. n = 90 cells.

Figure 8

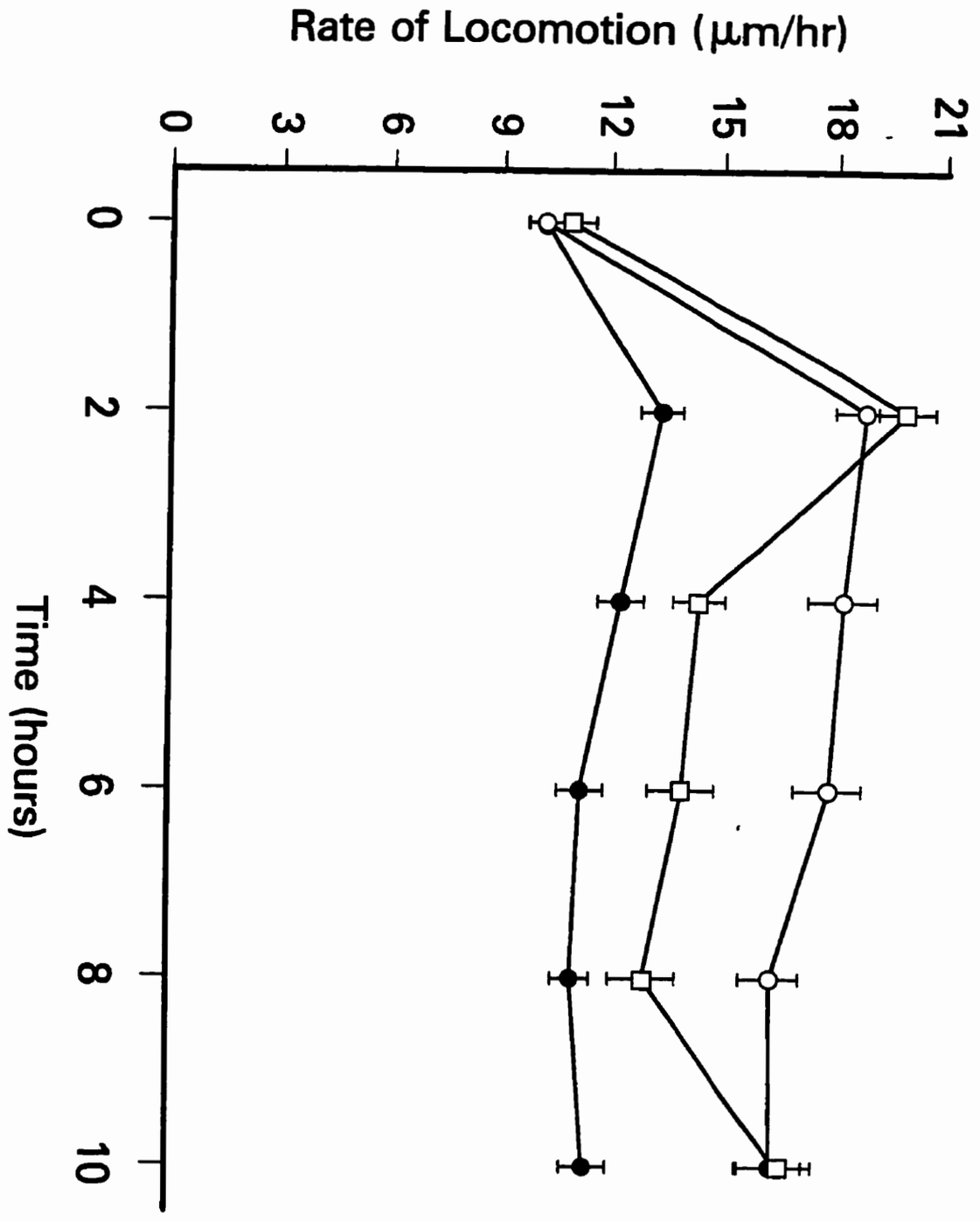


Figure 9. Microinjection of antiphosphotyrosine antibodies inhibits HA stimulated motility. C3 fibroblasts were microinjected with anti-phosphotyrosine antibodies or rabbit IgG control injection prior to HA (10 ng/ml) addition and motility was recorded for 2 h. Uninjected control cells (*A*) and cells injected with rabbit IgG (*B*) respond to HA with increased locomotion but cell injected with anti-phosphotyrosine antibodies (*C*) do not. Values represent the mean $\pm$ SEM.

Figure 9

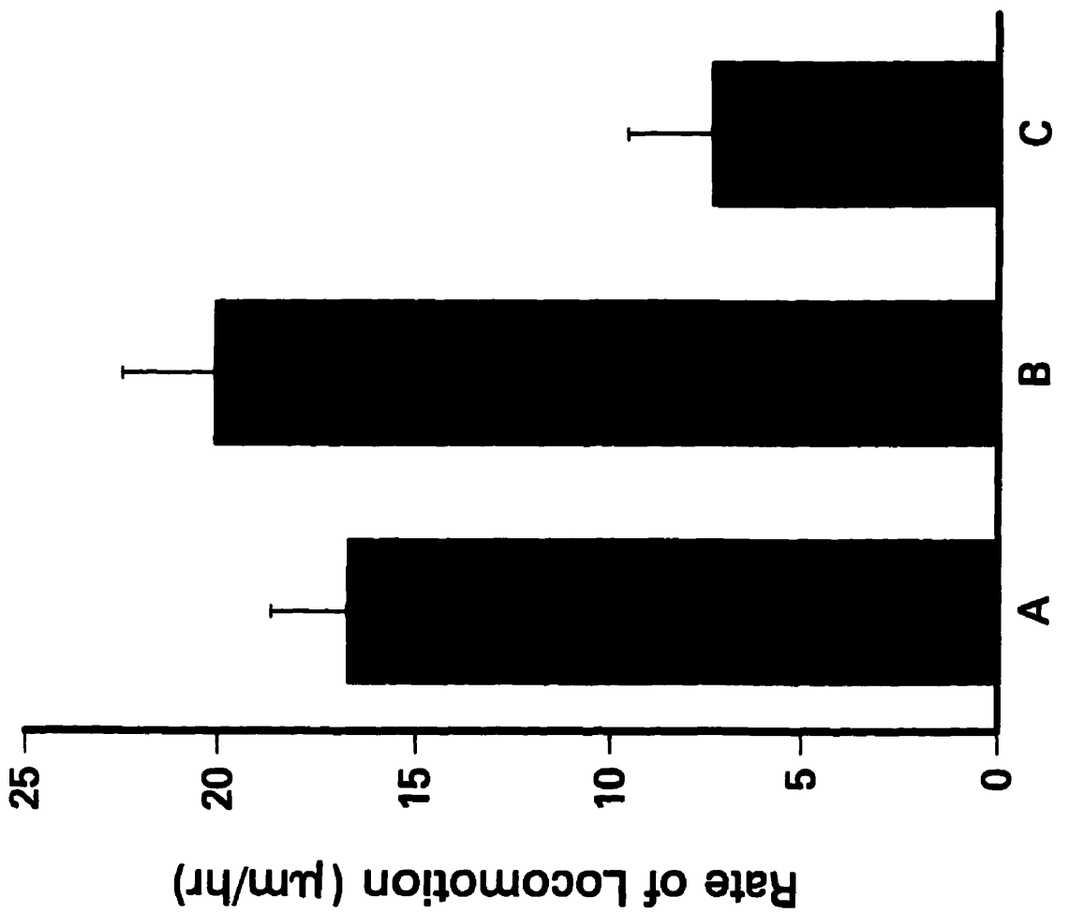


Figure 10. Immunofluorescent localization of vinculin after HA stimulation. C3 fibroblasts were incubated in the absence (*a-c*) or presence of 10 ng/ml HA for 1 min (*d-f*) or 15 min (*g-h*) prior to fixation and staining with anti-vinculin. Control cells have a few vinculin containing plaques. However with HA stimulation there is an increase number of vinculin containing plaques, then a disappearance of punctate vinculin staining. Mouse IgG control is shown (*i*). Size bar represents 26 μm.

Figure 10

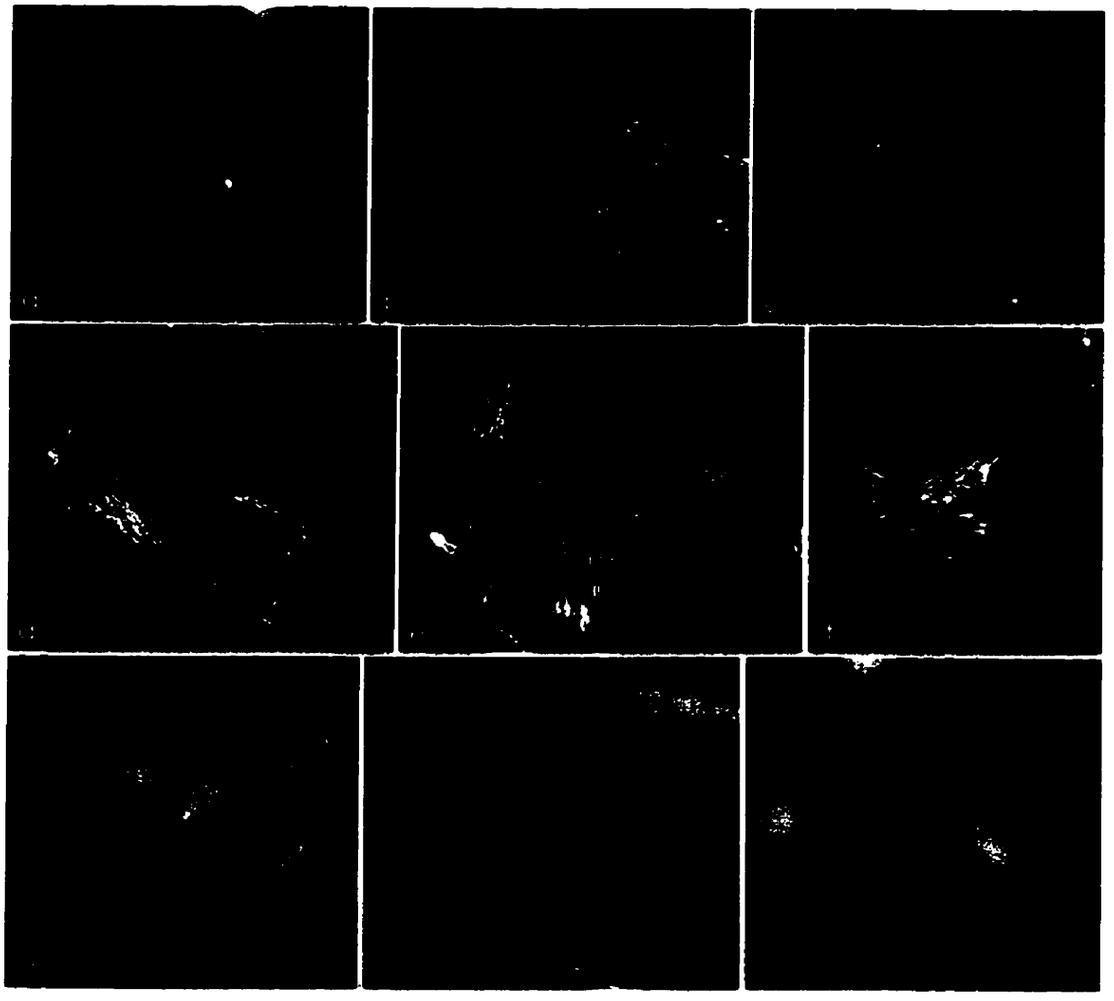


Figure 11. Double immunofluorescence for vinculin and phosphotyrosine following HA stimulation. C3 fibroblasts were incubated with 10 ng/ml HA for 1 min prior to fixation and staining with mouse anti-vinculin (*a, b, c*) and rabbit anti-phosphotyrosine (*e, f, g*). Vinculin and phosphotyrosine staining colocalize in some (open arrows) but not all (solid arrows) areas. Mouse IgG (*d*) and rabbit IgG (*h*) controls are shown. Size bar represents 8.5 μm.

Figure 11

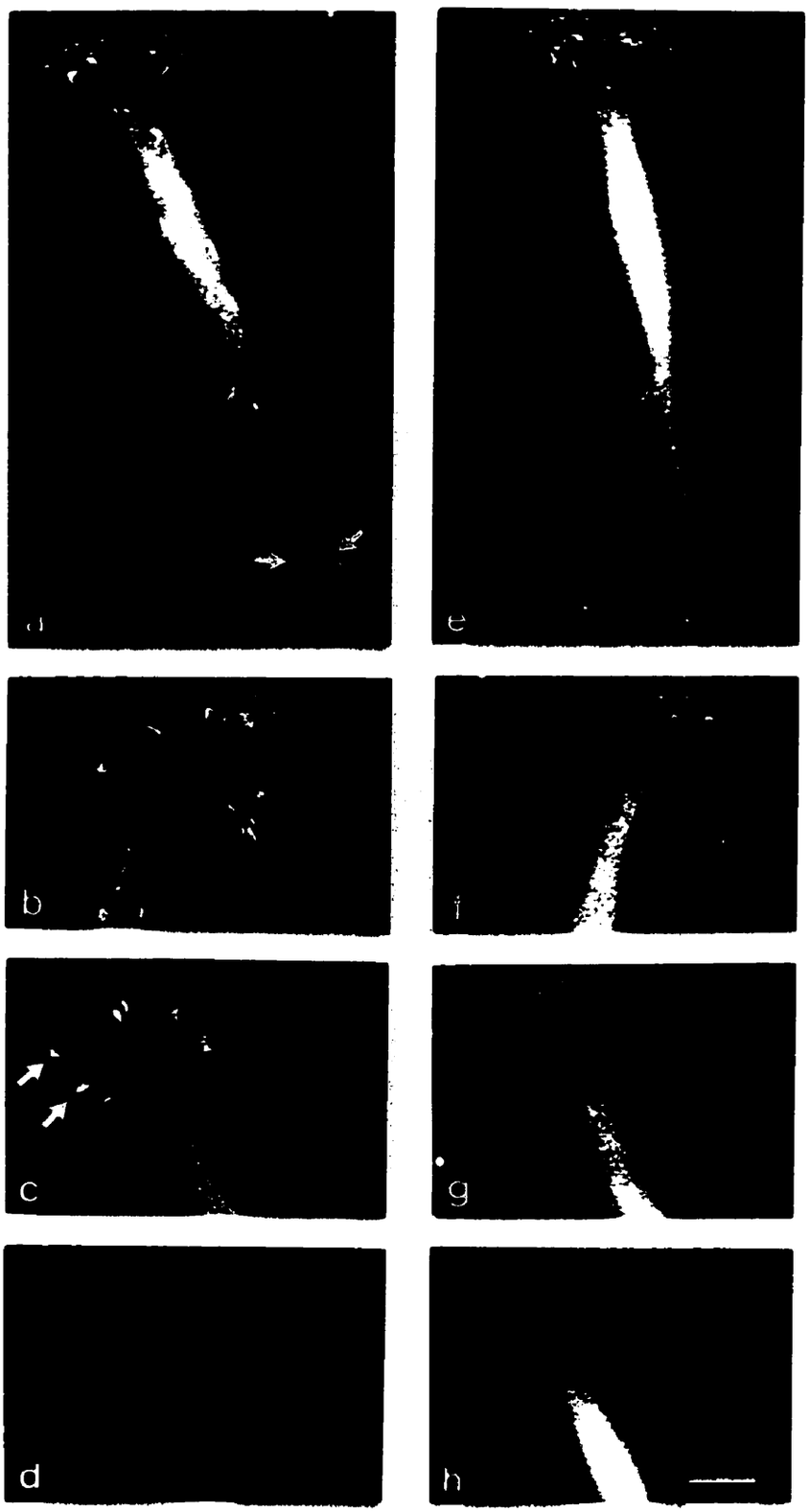
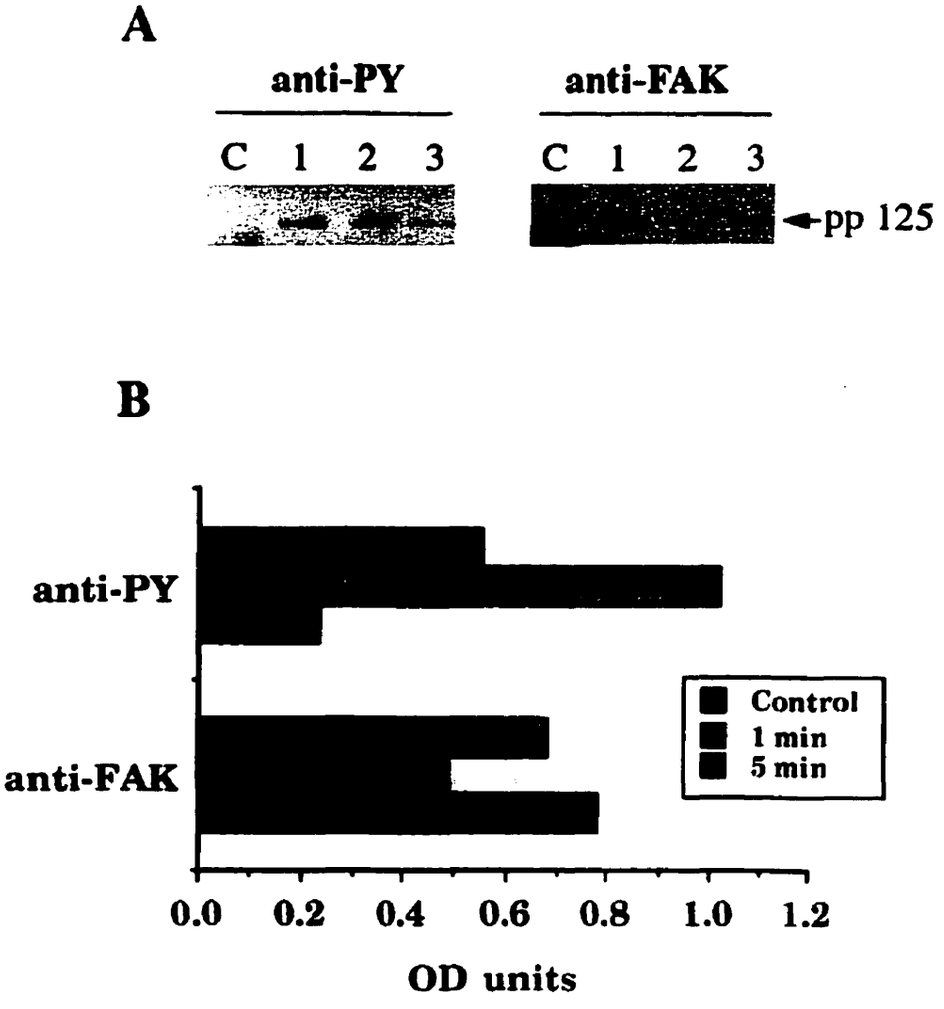


Figure 12. The effect of HA addition on the tyrosine phosphorylation of pp125^{FAK}. *A*. C3 fibroblasts were incubated in the absence (lane *1*) or presence of HA (10 ng/ml) for 1 min (lane *2*) or 5 min (lane *3*) prior to cell lysis. Immunoprecipitation was performed with anti-FAK or mouse IgG control (lane *C*), followed by SDS-PAGE and anti-phosphotyrosine (4G10) immunoblotting and stripping and reprobing with anti-FAK. *B*. Densitometry of both blots reveals that after HA addition there is a transient increase, followed by a decrease in pp125^{FAK} phosphorylation.

Figure 12


A
anti-PY
anti-FAK
C 1 2 3
C 1 2 3
pp 125
B
anti-PY
anti-FAK
Control
1 min
5 min
0.0 0.2 0.4 0.6 0.8 1.0 1.2
OD units

Chapter 2

Overexpression of the Hyaluronan Receptor RHAMM is Transforming and is also Required for H-*ras* Transformation

C.L. Hall, B. Yang, X. Yang, S. Zhang, M. Turley, S. Samuel, L.A. Lange, C. Wang, G.D. Curpen, R.C. Savani, A.H. Greenberg and E.A. Turley

Cell, Volume 82, July 14 1995, pages 19-28

Preface

Chapter 1 illustrated the importance of HA:RHAMM signaling in *ras*-transformed fibroblast motility. Because of the relationship between RHAMM, *ras* and cell locomotion (Turley *et al.*, 1991; Hardwick *et al.*, 1992; Hall *et al.*, 1994), we wished to determine if RHAMM alone could signal elevated cell motility in the absence of mutant activated *ras*. Furthermore, since RHAMM is necessary for elevated cell locomotion induced by *ras*, we wished to determine whether any of the other cell properties first attributed to activated mutant *ras*, such as morphological transformation, tumorigenic behaviour and metastatic capabilities, are due to RHAMM expression. Our goal of examining the effect of RHAMM overexpression

on transformation and metastasis has a strong foundation in the literature based on the implicated role for HA in transformation and metastasis (see Turley, 1984; Toole *et al.*, 1989; Laurant & Fraser, 1992; Knudson & Knudson, 1993; Sherman *et al.*, 1994; Hall & Turley, 1995). HA has been found to be enriched in tumor stroma and tumor cells have been shown to produce more HA and express higher levels of HA receptors than their non-metastatic counterparts (see Gunthert, 1993; Knudson & Knudson, 1993; Sherman *et al.*, 1994). In addition, HA has been found to stimulate the invasion of tumor cells (Turley *et al.*, 1987, Wang & Turley, 1994), and not only RHAMM, but another HA receptor, CD44 (Hart *et al.*, 1991; Thomas *et al.*, 1992) has been implicated in tumor cell locomotion. Overexpression of CD44 increases primary and secondary tumor growth (Sy *et al.*, 1991), a property that requires HA binding capabilities (Bartolazzi *et al.*, 1994). Furthermore, the expression of a variant form of CD44 on tumor cells yields metastatic capabilities (Gunthert *et al.*, 1991). Thus, we first wished to examine the effects of RHAMM overexpression on cell motility and transformation. Next, because of the connection between hyaluronan and tumorigenesis, we wished to determine if the effects of RHAMM overexpression required hyaluronan binding.

During this study, it is important to consider other ECM molecules and their receptors which have also emerged as regulators of signaling pathways involving *ras*. In particular, integrin mediated adhesion has been shown to recruit components of the *ras* pathway to focal adhesions where they interact with $pp125^{FAK}$ (Schlaepfer *et al.*, 1994). In addition, other cytoplasmic small GTP-binding proteins in the *ras*

family, namely rac and rho, have been shown to be involved in a *ras*-transformation pathway and to be required for *ras*-transformation (Khosravi-Far *et al.*, 1995; Prendergast *et al.*, 1995; Qiu *et al.*, 1995). Rac and rho are regulators of the cytoskeleton, focal adhesions and membrane extension (Nobes & Hall, 1995). While these *ras* family proteins may influence *ras* function through shared regulatory proteins, it is also likely that they influence *ras* function by altering cytoskeletal structures and affecting the association of key proteins with the cytoskeleton. Since RHAMM regulates cytoskeletal reorganization, focal adhesion turnover and lamellar extension (Chapter 1-Hall *et al.*, 1994; Entwistle *et al.*, 1996), RHAMM may also be required for *ras* function in a similar manner to integrins, rac and rho. Thus, we wished to determine how *ras* performed in the absence of functional RHAMM in terms of cell locomotion, transformation, cytoskeletal/focal adhesion organization and signal transduction in response to HA.

Specific Objectives

1. To overexpress the murine RHAMM gene and an isolated RHAMM cDNA, RHAMM1v4 (here; Entwistle *et al.*, 1995), in the fibroblast cell lines 10T½ and NIH 3T3. Further, to examine the effects of RHAMM overexpression on cell motility, cell morphology, and transformation.
2. To determine if the effects of RHAMM overexpression require hyaluronan binding by overexpressing RHAMM mutated in its HA binding domains.
3. To determine the role of RHAMM in *ras*-transformation by overexpressing

a "dominant negative" RHAMM in *ras*-transformed fibroblasts and by expressing mutant activated *ras* in cells expressing low levels of RHAMM due to anti-sense expression.

4. To determine the effects of dominant negative RHAMM expression on cell motility, cytoskeletal organization and signal transduction in response to HA in *ras*-transformed cells.

Contribution

This chapter appears as a published paper in Cell as referenced above. This multi-author publication is a collaboration among the laboratories of Dr. Turley, Dr. Greenberg and Dr. Savani. Equal contribution to this work is shared by myself (CLH) and BY. Briefly, the overexpression of the RHAMM gene by transfection into fibroblast cell lines were done by BY, SZ, SS, and GDC. Expression of a dominant suppressor mutant of RHAMM in *ras*-transformed cells was carried out by BY and the introduction of RHAMM antisense into fibroblasts was done by XY. Analysis of all of these cell lines were conducted by CLH, BY, XY, SZ, MT, LAL, CW and GDC. I conducted all of the signal transduction and focal adhesion work presented in Figure 3. In addition the data was compiled, researched and written by myself and EAT with direction and editing by EAT.

References

Bartolazzi A, Peach R, Aruffo A and Stamenkovic I. (1994). Interaction between CD44 and hyaluronate is directly implicated in the regulation of tumor development. *J. Exp. Med.*, **180**, 53-66.

Entwistle J, Hall CL and Turley EA. (1996). HA receptors: Regulators of signaling to the cytoskeleton. *J. Cell. Biochem.*, **61**,569-577.

Entwistle J, Zhang S, Yang B, Wong C, Li Q, Hall CL, A J, Mowat M, Greenberg AH and Turley EA. (1995). Characterization of the murine gene encoding the hyaluronan receptor RHAMM. *Gene*, **163**, 233-238.

Gunthert U. (1993). CD44: a multitude of isoforms with diverse functions. *Curr. Topics Microbiol. Immunol.*, **184**, 47-63.

Gunthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haumann I, Matzhu S, Wenzel A, Ponta H and Herrlich P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell*, **65**, 13-24.

Hall CL and Turley EA. (1995). Hyaluronan:RHAMM mediated cell locomotion and signaling in tumorigenesis. *J. Neuro-Oncol.*, **26**, 221-229.

Hall, CL, Wang C, Lange LA and Turley EA. (1994). Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity. *J. Cell Biol.*, **126**, 575-588.

Hall CL, Yang B, Yang X, Zhang S, Turley M, Samuel S, Lange LA, Wang C, Curpen GD, Savani RC, Greenberg AH and Turley EA. (1995). Overexpression of the hyaluronan receptor RHAMM is transforming and is

also required for H-ras transformation. *Cell*, **82**, 19-29.

Hardwick C, Hoare K, Owens R, Hohn HP, Hook M, Moore D, Cripps V, Austen L, Nance DM and Turley EA. (1992). Molecular cloning of a novel hyaluronan receptor that mediated tumor cell motility. *J. Cell Biol.*, **117**, 1343-1350.

Hart IR, Birch M and Marshall JF. (1991). Cell adhesion receptor expression during melanoma progression and metastasis. *Cancer Met. Rev.*, **10**, 115-128.

Khosravi-Far R, Solski PA, Clark GJ, Kinch MS and Der CJ. (1995). Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. *Mol. Cell. Biol.*, **15**, 6443-6453.

Knudson CB and Knudson W. (1993). Hyaluronan-binding proteins in development, tissue homeostasis and disease. *FASEB J.*, **7**, 1233-1241.

Laurant TC and Fraser JRE. (1992). Hyaluronan. *FASEB J.*, **6**, 2397-2404.

Nobes CD and Hall A. (1995). Rho, rac and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*, **81**, 53-62.

Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF and Der CJ. (1995). Critical role of Rho in cell transformation by oncogenic Ras. *Oncogene*, **10**, 2289-2296.

Qiu R-G, Chen J, Kirn D, McCormick F and Symons M. (1995). An essential role for rac in ras transformation. *Nature*, **374**, 457-459.

Rozengurt E. (1995). Convergent signaling in the action of integrins, neuropeptides, growth factors and oncogenes. *Cancer Surveys*, **24**, 81-96.

Schlaepfer DD, Hanks SK, Hunter T and van der Geer P. (1994). Integrin-mediated signal transduction linked to ras pathway by GRB2 binding to focal adhesion kinase. *Nature*, **372**, 786-791.

Sherman L, Sleeman, J, Herrlich P and Ponta H. (1994). Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. *Curr. Opin. Cell Biol.*, **6**, 726-733.

Sy MS, Guo YJ and Stamenkovic I. (1991). Distinct effects of two CD44 isoforms on tumor growth *in vivo*. *J. Exp. Med.*, **174**, 859-866.

Thomas L, Byers HR, Vink J and Stamenkovic I. (1992). CD44H regulates tumor cell migration on hyaluronate-coated substrate. *J. Cell Biol.*, **118**, 971-977.

Toole BP, Munaim SI, Welles S and Knudson CB. (1989). The role and regulation of tumour-associated hyaluronan. *Ciba Found. Symp.: Biology of Hyaluronan*, **143**, 138-149.

Turley EA. (1984). Proteoglycans and cell adhesion: their putative role during tumorigenesis. *Cancer Met. Rev.*, **3**, 325-339.

Turley EA, Austen L, Vandeligt K and Clary C. (1991). Hyaluronan and a cell-associated hyaluronan binding protein regulate cell locomotion in ras-transformed cell. *J. Cell Biol.*, **112**, 1041-1047.

Turley EA, Tretiak M and Tanguay K. (1987). Effect of glycosaminoglycans and enzymes on the integrity of human placental amnion as a barrier to cell

invasion. *J. Natl. Cancer Inst.*, **78**, 787-795.

Wang C and Turley EA. (1994). RHAMM is required for the motility and invasion of breast cancer cells in vitro. (Abstract) *J. Cell. Biochem.*, Suppl. **18D**, 240.

OVEREXPRESSION OF THE HYALURONAN RECEPTOR RHAMM IS TRANSFORMING AND IS ALSO REQUIRED FOR H-*ras* TRANSFORMATION

Christine L. Hall*, Baihua Yang*, Xuiwei Yang, Shiwen Zhang, Maureen Turley, Shanti Samuel, Laurie A. Lange, Chao Wang, Genevieve D. Curpen, Rashmin C. Savani, Arnold H. Greenberg, and Eva A. Turley

Manitoba Institute of Cell Biology, and
Department of Pediatrics, University of Manitoba
Winnipeg, Manitoba, Canada, R3E 0V9

* Equal Contribution

SUMMARY

Overexpression of the RHAMM gene by transfection into fibroblasts is transforming and causes spontaneous metastases in the lung. H-*ras*-transformed fibrosarcomas transfected with a dominant suppressor mutant of RHAMM exhibit a so-called revertant phenotype and are completely non-tumorigenic and non-metastatic. Conversely, fibroblasts stably expressing low levels of RHAMM as a result of antisense transfection are resistant to *ras* transformation. Collectively, these results indicate that RHAMM acts downstream of *ras*. The loss of functional RHAMM ablates signaling within focal adhesions, in particular changes in FAK phosphorylation, and as a result these focal adhesions are unable to turnover in response to hyaluronan. These results provide the first evidence of the oncogenic potential of a novel extracellular matrix receptor and establish a functional link between transformation by *ras* and signaling within focal adhesions that are required for transformation by this oncogene.

Running Title: **RHAMM is required for *ras*-transformation**

INTRODUCTION

Extracellular matrix (ECM) molecules, their receptors and gene products that modify the ECM are able to directly influence cell characteristics such as growth and motility (Ratner, 1992; Damsky and Werb, 1992; Hynes, 1992; Ruoslahti, *et al.*, 1994, Lin and Bissell, 1993; Jones *et al.*, 1993), to regulate cellular responses to growth factors and cytokines (Noble *et al.*, 1993; Chong *et al.*, 1992; Hiro *et al.*, 1986), and to modify the transformed state. Many of these extracellular matrix receptors and their ligands can regulate the invasive/metastatic phenotype in tumorigenic cells. Thus, overexpression of $\alpha_4\beta_1$ integrin inhibits invasion during metastasis (Qian *et al.*, 1994) while overexpression of the urokinase receptor enhances invasion (Kariko *et al.*, 1993). A regulator of metalloproteinases, TIMP-1, inhibits invasion of metastatic cells (Khoka *et al.*, 1992; Alexander and Werb, 1992). Other ECM receptors, however, partially modify the transformed state of cells. Thus $\alpha_5\beta_1$ integrins partially restore contact inhibition and anchorage dependent growth properties of the tumorigenic CHO cells (Giancotti and Ruoslahti, 1990), while overexpression of thrombospondin (Castle *et al*, 1993) results in the development of serum independent growth. However, none of these ECM receptors have been previously shown to be frankly transforming.

The hyaluronan (HA) receptor RHAMM signals elevated cell locomotion via a transient tyrosine phosphorylation within focal adhesions which results in their turnover in *ras* transformed cells (Hall *et al*, 1994). RHAMM is regulated by growth factors such as TGF-β_1 and its expression is necessary for TGF-β_1 stimulation of

fibrosarcoma cell motility (Samuel *et al.*, 1993). We present here that, in contrast to other ECM receptors, overexpression of the HA receptor RHAMM (Hardwick *et al.*, 1992) is both transforming and able to generate a "metastatic" phenotype in which the subcutaneous tumors of RHAMM-transfected cells spontaneously metastasize and these cells also form lung colonies after inoculation in the tail vein. The ability of RHAMM to transform fibroblasts indicates that this receptor functions in a fundamentally different manner than other characterized ECM receptors. In view of the elevated expression of RHAMM in *ras*-transformed cells (Turley *et al.*, 1991) and its importance in the locomotion of these cells (Hardwick *et al.*, 1992), we have also investigated the role of this receptor in *ras*-transformation. We show here that disruption of RHAMM function by antisense RNA expression or by a dominant negative mutant of RHAMM profoundly reduces the transforming properties of the *ras*-oncogene. We also link the ability of the dominant negative RHAMM mutation to ablate *ras* transformation to an effect on signaling via the focal adhesion kinase, $pp125^{FAK}$ within focal adhesions.

RESULTS

Overexpression of RHAMM in fibroblasts induces transformation

Since it had been previously shown that RHAMM expression was elevated in *ras*-transformed cells (Turley *et al.*, 1991; Hardwick *et al.*, 1992; Samuel *et al.*, 1993), the contribution of RHAMM to the transformation process was investigated by its overexpression in non-senescing fibroblasts. 10T½ fibroblasts were

transfected with either a genomic RHAMM clone λ4 (Hardwick *et al*, 1992; Entwistle *et al.*, submitted), or a RHAMM cDNA encoding an isoform common to *ras*-transformed cells (RHAMM 1v4, Zhang *et al.*, submitted). Both transfections were chemically selected and cell lines cloned. Five clones that overexpressed RHAMM, as determined by Western blot analysis of cell lysates (Table 1) and Northern blot analysis of RHAMM RNA (data not shown), were selected from each transfection. These selected clones were further characterized and their properties are summarized in Table 1. Cells transfected with the RHAMM gene overexpressed a 73 kDa protein consistent with the predicted size of RHAMM (data not shown; Entwistle *et al.*, submitted). The same cells displayed an increase in a 4.2 kb message, the size of the major RHAMM mRNA transcript (data not shown; Hardwick *et al.*, 1992). The presence of the transfected RHAMM gene in all clones was confirmed by PCR detection of plasmid arms (data not shown). The selected clones overexpressed products of the RHAMM gene at the cell surface as determined by FACS analysis (Table 1). Transfected cells appeared morphologically transformed in that they were rounded, showed no evidence of contact inhibition and possessed few focal adhesions (data not shown). Moreover, they exhibited a high nuclear overlap index comparable to that of *ras*-transformed cells (Table 1) and formed foci in monolayer culture unlike the vector controls or the 10T½ parent cell line (Table 1). Furthermore, transfected cells reached culture confluence at a 3-4 fold higher cell number relative to vector control or parent line controls (data not shown). The rate of random locomotion of the transfected cells was 2-3 fold higher than

control cell lines, a rate comparable to the *ras*-transformed C3 cell line (Table 1). This high motility was maintained at sub-confluence and was blocked by anti-RHAMM antibodies (Table 1). The rate of growth of RHAMM-transfected cell lines was less than that of *ras*-transformed C3 cells, and in fact, identical to the control cells until confluence halted growth of the latter. Nevertheless, RHAMM transfected cells grew in an anchorage-independent fashion in soft agar (data not shown). Similar results on cell behaviour were obtained after transfection of the RHAMM 1v4 cDNA in a pHβAP-3P-Neo vector into 10T½ or 3T3 fibroblasts (Table 1). Transfection of RHAMM 1v4 cDNA containing mutated HA binding domains (Figure 1) had no effect on morphology, contact inhibition or motility (Table 1).

To determine whether cells transfected with the RHAMM λ4 gene were tumorigenic, 1-5 x 10^6 cells were injected subcutaneously into the right hind leg of syngeneic mice. Fibrosarcomas formed within three weeks; no tumors formed in mice injected with vector transfected or 10T½ parental control cells (Table 1). Tumors derived from the transfected cells were identical histologically to those formed by *ras*-transformed cells and expressed high levels of RHAMM as seen immunohistochemically. Further, in approximately 50% of animals bearing subcutaneous tumors, tumors spontaneously metastasized to the lung to form a mean of 5 nodules/lung (Table 1). The occurrence of tumor cells within these nodules was confirmed by histology. Cells injected into mice via the tail vein invaded lung tissue and formed metastatic nodules (Table 1). Cells transfected with RHAMM 1v4 cDNA were also tumorigenic and metastatic, but cells expressing the mutated

RHAMM 1v4, lacking HA binding domains, were not (Table 1).

These results were repeated with NIH-3T3 fibroblast cell line. 5 x 10^6 transfected cells were inoculated subcutaneously into syngeneic mice and the presence of tumors was observed 3 weeks (Table 1).

Reversion of H-*ras*-transformation with a dominant suppressor mutant of RHAMM.

The importance of RHAMM for maintenance of the transformed phenotype of H-*ras*-transformed fibrosarcomas was examined by blockade of RHAMM function with a RHAMM cDNA mutated at its HA binding domains (Figure 1). Similar suppressor mutations have been previously prepared by mutating the kinase domain (Evans *et al.*, 1993) or deleting the cytoplasmic domains of other receptors (Kashles *et al.*, 1991). The present approach to functional ablation was taken since RHAMM is secreted, is localized at the cell surface and forms homodimers (Hardwick *et al.*, 1992; Klewes *et al.*, 1993). Furthermore, mutation of the HA binding domains in RHAMM destroys its ability to morphologically transform fibroblasts (Table 1). Collectively, these conditions have been found to be sufficient to construct suppressor mutations of such growth factor receptors as TGF-β_1 (Brand *et al* , 1993). The details of the amino acid substitutions in the HA binding domains of RHAMM are outlined in Fig. 1A. Loss of HA binding was confirmed using the mutated RHAMM fusion protein in a ligand blotting assay, the specificity of which has previously been demonstrated (Fig. 1A; Hoare *et al.*, 1993). *Ras*-transformed 10T½ fibroblasts (termed C3) were transfected with the mutated RHAMM and chemically

selected in hygromycin. Over 20 clones were selected, 25% of which displayed a flattened cell shape that was morphologically similar to 10T½ fibroblasts not transformed by *ras* (Fig. 2C). Three clones containing the mutated RHAMM protein were selected for further analysis and all three were found to overexpress RHAMM by 2-3 fold as determined by Western blot analysis (for examples, see Fig. 1B 2-4). Increased cell surface expression of RHAMM was detected by FACS analysis (Fig. 1D) using antibody A268 (Hardwick *et al.*, 1992), which specifically recognizes a peptide encoded in the RHAMM cDNA (aa $^{268\text{-}289}$) that is 5' to the mutated region of the protein (Fig. 1C). Despite their flattened morphology, all clones overexpressing mutant RHAMM exhibited levels of $p21^{ras}$ protein that were comparable to or higher than those seen in the H-*ras* transformed C3 fibrosarcoma cells transfected with vector only (Fig. 1C).

Despite the expression of high levels of activated r*as*, clones expressing the suppressor mutant of RHAMM (MR-C3-4D,-5B, -5C) more closely resembled the non-transformed 10T½ cells in their growth characteristics, locomotion rates and tumorigenic capabilities. Mutated RHAMM clones were contact inhibited and displayed a low nuclear overlap ratio comparable to the non-transformed 10T½ cell line (Fig. 2A-C). They had a lower saturation density than *ras*-transformed vector control cells (Fig. 2D) and showed suppressed rates of locomotion in comparison to transformed parental C3 fibrosarcoma cells (Table 2). In contrast to vector transfected controls, the cells expressing the dominant suppressor RHAMM failed to form foci in monolayer cultures (Fig. 2E) and did not form colonies in soft agar.

(Table 2). When injected subcutaneously into syngeneic mice, no tumors were detected after 6 months of observation (Fig. 2F), while vector controls and the *ras*-10T½ parent cell line formed large tumors within 3 weeks (Fig. 2F). In addition, the clones expressing mutated RHAMM did not develop tumors in the lung colonization assay for metastasis (Fig. 2G).

Expression of dominant suppressor RHAMM ablates signal transduction to focal adhesions.

We have previously shown that in *ras*-transformed fibroblasts RHAMM signals cell locomotion via a transient tyrosine phosphorylation pathway that targets focal adhesions (Hall *et al*, 1994). Stimulation of these cells results in two rapid and transient events, tyrosine phosphorylation of several protein bands and the formation of focal adhesions. Net dephosphorylation and focal adhesion turnover rapidly follow concomitant with elevated cell motility. Since expression of the dominant suppressor RHAMM inhibits cell locomotion, among other transformation dependent processes (Table 2), we examined the effect of this protein on signal transduction and focal adhesions. Vector transfected C3 cells responded to HA stimulation as previously reported, with a transient increase in the phosphorylation of several protein bands (p185, p125, p115, p85) followed by net dephosphorylation (Fig. 3A). In particular, the focal adhesion kinase, $pp125^{FAK}$, has been identified as a substrate in this pathway (Hall *et al.*, 1994). Transient tyrosine phosphorylation of this substrate, followed by dephosphorylation, coincidental with focal adhesion turnover, is observed in vector control cells (Fig. 3B). Tyrosine phosphorylation of FAK and

other protein bands in the dominant suppressor expressing cells (MR-C3-4D) remained constant over the stimulation time course (Fig. 3A, B). Thus, *ras*-transformed fibroblasts expressing the dominant suppressor RHAMM cannot utilize the signal transduction pathway that involves changes in FAK phosphorylation and that is required for transformation by *ras*.

Cells possessing stable focal adhesions move more slowly and are less tumorigenic (see Discussion). *Ras*-transformed fibroblasts normally have very few focal adhesions, but form temporary focal adhesions at the cell edge after HA stimulation (Vector control; Fig. 3C, panel a). On the contrary, the cells expressing the HA binding deficient RHAMM have numerous focal adhesions throughout the cell in the presence or absence of HA (Fig. 3C, panel b), thus resembling non-transformed 10T½ fibroblasts. Similarly, tyrosine phosphorylation levels in these cells are higher and largely occur in the focal adhesions (Fig. 3C, panel d). The HA stimulated vector control cells exhibit rapid onset of increased phosphotyrosine staining only at the lamellae edges and only at 1 min. after stimulation with HA, while the MR-C3-4D cells do not respond to HA but maintain high levels of plaque-like phosphotyrosine staining in the cell body and at the lamellae edges (Fig. 3C, panels c and d).

Antisense RHAMM confers resistance to transformation by mutant H-*ras*.

To determine whether fibroblasts with suppressed RHAMM expression could be transformed with *ras*, 10T½ cells were transfected with RHAMM cDNA in an antisense orientation. Two G418 resistant clones (termed OR1 and OR2) that

expressed 10 to 50% of the detectable RHAMM protein levels seen in vector controls were selected from over 60 clones with varying reductions of RHAMM expression (Yang, X. *et al*, in preparation). The two clones expressing low levels of RHAMM protein were identical in their properties. The presence of RHAMM antisense and the reduction or lack of RHAMM message was demonstrated by RT-PCR and confirmed by Southern blot hybridization (Yang X. *et al.*, in preparation). The two transfected cell lines, the vector control and the parent 10T½ cells were then grown to confluence and transfected with an activated H-*ras*. The vector control and the parental line formed multiple large and small foci after three weeks in culture (Fig. 4A-B), whereas cultures containing the OR1 and OR2 constructs formed no such foci (Fig. 4C-D). Furthermore, these clones, unlike *ras*-transformed controls, retained a flattened morphology. All *ras*-transfected cultures expressed $p21^{ras}$ protein (Fig. 4E) at levels similar to those of the *ras*-transformed cell line C3 (compare with Fig. 1C).

DISCUSSION

We demonstrate here that the HA receptor RHAMM, a GPI-linked-plasma membrane protein (Klewes *et al.*, manuscript in preparation) is not only transforming but that its expression is sufficient to generate a metastatic fibrosarcoma. To our knowledge, this is the first report of an ECM receptor that is capable of transforming fibroblasts when overexpressed. In addition, RHAMM also appears to be necessary for initiating and maintaining transformation induced by other oncogenes such as *ras*.

Since *ras* transformed cells expressing nonfunctional RHAMM are unable to signal and turnover focal adhesions, these results also imply a direct role for RHAMM in signaling within these cytoskeletal structures in the transformation process downstream of *ras*.

The transforming capability of RHAMM and the inability of RHAMM mutated in its HA binding domains to transform cells demonstrate for the first time, a causal role for HA in the transformation process . The observation that mutation of RHAMM in its HA binding domains ablates *ras* transformation provides further evidence connecting HA to transformation. Hyaluronan, a ubiquitous glycosaminoglycan, has previously been linked with both transformation and the metastatic process (Turley, 1992; Knudson and Knudson, 1993; Fraser and Laurent, 1993) since it is often enriched in tumor-associated stroma (Turley and Tretiak, 1984) and its presence facilitates melanoma invasion across chorioallantoic membranes (Turley *et al.*, 1987). Highly metastatic tumor cells produce more HA (for review, see Knudson and Knudson, 1993) and express higher levels of HA receptors (Gunthert, 1993; Haynes, 1992; Hardwick *et al.*, 1992) than less metastatic counterparts. Moreover, overexpression of one of the isoforms of the HA receptor CD44 increases primary and secondary tumor growth (Sy *et al.*, 1991), a property that requires the HA binding domain of this receptor (Bartolazzi *et al*, 1994). As well, the V6 isoform of CD44 bestows a metastatic phenotype on transformed cells (Gunthert *et al.*, 1991), although it is not known whether this property is dependent upon the HA binding capability of this isoform.

As noted in the introduction, other ECM receptors and ECM molecules (Hynes, 1992; Gunthert *et al.*, 1991; Stetler-Stevenson *et al.*, 1993; Schwartz, 1993; Behrend *et al*, 1994) have also been linked to the regulation of the invasive properties of cells, or to the partial modification of the transformation process itself. It is important to note, however, that none of these molecules have been demonstrated to be transforming by themselves, implying that RHAMM has a unique role in orchestrating events that are essential for transformation to occur. These events include the ability of RHAMM to signal, via focal adhesions, alterations in the cytoskeleton and elevated cell locomotion.

Focal adhesions are sites of ECM receptor-cytoskeletal interactions where second messenger signaling commonly occurs in response to the ECM and some growth factor receptors. These structures, also described as signal transduction units (Lo and Chen, 1994; Zachary and Rozengurt, 1992), are believed to be critical for the regulation of growth and cell motility (Woods and Couchman, 1988; Burridge *et al.*, 1988). They have previously been linked to transformation in that very few focal adhesions are present in tumor cells (Burridge *et al.*, 1988) and focal adhesion assembly promoted by overexpression of the focal adhesion components, $\alpha_5\beta_1$ integrin, tensin or vinculin, cause partial reversal of transformation (Giancotti and Ruoslahti, 1990; Varner *et al.*, 1992; Lo and Chen, 1994). Conversely, antisense ablated expression of the focal adhesion protein vinculin promotes transformation dependent properties (Rodriguez-Fernandez *et al.*, 1993) and selective targeting of truncated v-*src* to focal adhesions, but not to the nucleus or to the cytoplasm, is

sufficient for transformation by this oncogene (Leibl and Martin, 1992). We have previously noted that in *ras*-transformed fibroblasts, unlike their parental counterparts, HA regulates cell motility via RHAMM by signaling transient protein tyrosine phosphorylation within focal adhesions (Hall *et al*, 1994). In this signaling pathway the focal adhesion kinase, FAK, is transiently phosphorylated followed by net dephosphorylation and focal adhesion turnover leading to the initiation of cell locomotion (Hall *et al*., 1994). Cells overexpressing RHAMM resemble *ras*-transformed fibroblasts and have elevated cell locomotion and focal adhesion loss, as well as tumorigenic and metastatic potential. Conversely, expression of a dominant suppressor mutant of RHAMM reverts transformation induced by *ras* and stabilizes focal adhesions (X. Yang *et al.*, in preparation). This HA receptor targets focal adhesions and likely acts downstream of *ras* or via a parallel pathway that converges at the level of *ras*.

FAK has been implicated in several signaling pathways that involve ECM receptors, as well as in transformation (Schaller *et al*, 1992; Calalb *et al*., 1994). The stimulation of activation by growth factors, neuropeptides and the ECM is associated with cell attachment, focal adhesion assembly and stress fiber formation (Schaller and Parsons, 1994; Zachary and Rozengurt, 1992). Cell adhesion via integrins can stimulate the formation of signaling complexes containing FAK, c-*src*, Grb-2 and Sos (Schlaepfer *et al.*, 1994), thus providing a mechanism for the regulation of the *ras*/MAP kinase signaling cascade downstream of FAK phosphorylation. On the contrary, transient FAK phosphorylation, followed by net FAK dephosphorylation

has been associated with elevated cell migration (Hall *et al.*, 1994; Matsumoto *et al.*, 1994) and, hence, may represent the mechanism by which *ras* and RHAMM regulate elevated cell motility. We show here that a dominant suppressor mutation of RHAMM that ablates *ras* transformation collectively prevents HA binding, HA triggered FAK signaling, focal adhesion turnover and cell motility. Thus, while integrin and growth factor regulated FAK phosphorylation would appear to be upstream of *ras* signaling, our results indicate that FAK dephosphorylation, regulated by RHAMM, acts downstream of mutant *ras* and is an event that is required for transformation by this oncogene. While our results specifically suggest that the constitutive ability to turnover focal adhesions is a requirement for *ras* transformation, this event by itself is unlikely to be sufficient for transformation to occur. Hence, the overexpression of the ECM components, thrombospondin or tenascin reduce focal adhesion assembly (Borsi *et al*, 1992; Murphy-Ullrich et al., 1989) but both are only partially transforming, merely enhancing serum and anchorage-independent growth of immortalized cell lines (Castle et al., 1993; Murphy-Ullrich et al., 1991; Werle-Haller and Chiquet, 1993). Also, loss of focal adhesions induced by the overexpression of a truncated *ras*-GAP has little effect on growth potential (McGlade et al., 1993). The regulation of focal adhesion turnover, probably effected by dephosphorylation of FAK, is likely required for transformation to occur but is unlikely to account for the tumorigenic/metastatic properties. Thus, overexpression of RHAMM must result in transformation by altering additional cell characteristics.

Signaling via tyrosine phosphorylation has previously been identified as an event that can lead to transformation and since RHAMM transiently activates protein phosphorylation, such signaling may ultimately lead not only to focal adhesion turnover but also to selective expression of genes that act collectively through *ras* to transform the cell. Previous reports link signal transduction pathways mediated by other ECM receptors (Damsky and Werb, 1992; Hynes, 1992; Juliano and Haskell, 1993; Schwartz, 1993) to changes in gene expression (Jones *et al.*, 1993). Mammary epithelial cells produce tissue specific proteins in response to basement membranes (Howlett and Bissell, 1993), while laminin, fibronectin and other ECM components regulate stromelysin, collagenase (Werb *et al.*, 1990; Shapiro *et al.*, 1993; Saarialho-Kere *et al.*, 1993) and other genes (Jones *et al.*, 1993). Hyaluronan has also previously been reported to regulate the expression of several cytokine genes in part via CD44 (Noble *et al.*, 1993; Chong *et al*, 1992). It is likely therefore that other genes are also regulated by RHAMM.

In summary, we have demonstrated that overexpression of RHAMM is transforming and that ablation of RHAMM expression or its function prevents *ras*-transformation. We also provide the first direct evidence of a role for signaling within focal adhesions for maintaining *ras* transformation.

EXPERIMENTAL PROCEDURES

Cell culture and cell lines

The previously established murine fibroblast cell lines 10T½, NIH 3T3, and the H-*ras*-10T½ CIRAS-3 (C3; Egan *et al.*, 1987) were utilized for the various transfection and tumorigenic studies. The cells were maintained at 37°C in 5% CO_2 in DMEM growth media (GIBCO BRL) supplemented with 10% fetal calf serum (Invitrogen). Cells were subcultured using trypsin/EDTA (0.25% Difco bactotrypsin, 2 mM EDTA) prior to reaching confluence. To determine culture density, 5 x 10^4 cells were added to each of 24 wells in 1 ml DMEM supplemented with 10% FCS and 0.6 mg/ml geneticin. Media was changed every 3 days and at each time point cells were released from the substratum with 0.25% trypsin and counted with a Coulter counter.

Preparation of DNA Constructs

The full length RHAMM gene was isolated from a 3T3 cell genomic library and is described elsewhere (Entwistle *et al.*, submitted). The genomic RHAMM clone, λ4, was harboured in EMBL3 phage arms for transfection. RHAMM cDNAs, mutated cDNAs and antisense cDNAs (Hardwick *et al.*, 1992; Entwistle *et al.*, submitted) were cloned into the pHβAPr-3p-Neo expression vector (Gunning *et al.*, 1987). The cDNAs mutated in their HA binding domains have been previously described (Yang *et al.*, 1994). Briefly, mutagenesis of specific basic amino acids in both of the HA binding domains of RHAMM was accomplished in two steps. First, site-directed mutations were confined to basic amino acids K^{405} and K^{409}. Next, the

basic amino acids[430,432] were mutated using the RHAMM cDNA generated in step 1. The mutations abolish HA binding of the resulting protein (Fig. 1; Yang *et al.*, 1994). The RHAMM antisense cDNA was produced by PCR of the entire coding region (amino acids 1 to 477; Hardwick *et al.*, 1992) and 172 nucleotides of the 3' flanking sequence. The fragment cloned into the expression vector in the antisense orientation was sequenced for confirmation.

Transfection with Genomic RHAMM in RHAMM cDNAs

The RHAMM λ4 clone in EMBL3 phage arms was co-transfected with PSV_2 plasmid into 10T½ fibroblasts using calcium phosphate. RHAMM transfected cells were selectively grown in growth media containing 0.6 mg/ml G418 for 3 weeks; colonies were cloned by selective trypsinization; and 15 clones were isolated. Four clones (λ4-2, -6, -10, and -12-10T½) that exhibited stable integration of the RHAMM gene by Southern analysis and overexpression the RHAMM protein by immunoblot analysis were selected for further study.

10T½ and NIH 3T3 cell lines were transfected with either the RHAMM 1v4 cDNA construct (Entwistle *et al.*, submitted; Zhang *et al.*, manuscript in preparation) in the pHβApr ^{3}P-Neo expression vector (Gunning *et al.*, 1987) or the RHAMM 1v4 cDNA mutated construct as described above. To produce stably transfected cell lines, fibroblasts were transfected using lipofectin (Gibco BRL) according to manufacturer's instructions and cells were selected in G418. Clones were then selected as above and tested for overexpression using Western analysis (Zhang *et al.*, manuscript in preparation). Based on their overexpression and representative

morphology, the following clones were used in this study: vector transfected cells, RHAMM 1v4 overexpressing clones (cDNA 5-10T½ and cDNA-3T3) and a mutated RHAMM 1v4 clone (MR10T½-4).

For dominant negative transfections, C3 cells were stably transfected with the mutated RHAMM cDNAs as above, except that the hygromycin gene was included in the pHβAPr ^{3}P-Neo vector. Cells were selected in hygromycin and geneticin (to maintain *ras* insert) then cloned. Clones that exhibited a flattened morphology (45% of clones) were selected, then analyzed for overexpression of RHAMM using a Western transblot assay. Three high expressing clones were selected.

RHAMM cDNA (Hardwick *et al.*, 1992) was used as a PCR template to generate a 1.7-kb fragment containing the entire coding region (amino acid 1 to 477) and 172 nucleotides of 3' flanking sequence. The fragment was cloned in an antisense orientation into the pHβApr-3p-Neo expression vector (Gunning *et al.*, 1987) and sequenced for confirmation. To produce stably transfected cell lines, 10T½ fibroblasts were transfected using lipofectin with RHAMM antisense plasmid constructs according to the manufacturer's instructions (Gibco BRL, Gaitherburg, MD, USA). Briefly, 1-2 x 10^5 of the 10T½ cells were seeded into 60 mm tissue culture dishes and cultured in growth medium containing DMEM and 10% fetal bovine serum. After reaching 50-70% confluence, cells were transfected with 10-20 μg of RHAMM plasmid and selected in G418 then cloned. Low production of RHAMM was detected by Western analysis and RT-PCR.

Cell Lysis and Immunoblot Analysis

C3 cell cultures were exposed to HA (10 ng/ml; Hall *et al.*, 1994) or control treatments for various time periods at 37°C, and then placed on ice. Culture media was removed, the plates rinsed with cold PBS (2.7 mM KCL, 1.1 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4; pH 7.4) containing 250 μM sodium orthovanadate and the cells were lysed with ice cold RIPA lysis buffer (25 mM tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA) containing 10 μg/ml leupeptin, 10 μg/ml Aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate (all chemicals from Sigma). Lysates were scraped into micro-centrifuge tubes and after 10 min on ice were centrifuged at 13,000 rpm for 15 min at 4°C (Heraus Biofuge 13, Baxter Diagnostics Corporation, Mississauga, Ontario). Protein concentrations of the supernatants and BSA standards were determined using the DC protein assay (Bio-Rad Laboratories, Rockville Center, NY) and duplicate samples containing 20 μg of protein each along with prestained molecular weight markers (Sigma) were separated by SDS-PAGE (12% gel; Laemmli, 1970). The proteins on the gels were either electrophoretically transferred to nitrocellulose membranes (Bio-Rad) or stained with Coomassie blue to check for equal loading. Additional protein-binding sites on the nitrocellulose membranes were blocked with 5% defatted milk in TBS (50 mM Tris HCl, pH 7.4, 200 mM NaCl), and then the membranes were incubated with anti-phosphotyrosine mAb (1 μg/ml 4G10 [UBI] in 1% defatted milk/TBS) or anti-RHAMM antibody (Yang *et al.*, 1994, 1:200) for 2 h at room temperature on a rotator (Nutator; Becton Dickinson and Company,

Parsippany, NJ). The membranes were washed four times in 0.05% Tween-20/TBS before incubation with peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000 dilution in 1% milk/Tween-TBS; Sigma) for 1 h at room temperature. After washing, blots were developed using ECL Western blotting detection system (Amersham International plc, Amersham, UK) according to manufacturer's instructions. To establish antibody specificity, parallel blots were probed with anti-phosphotyrosine that had been preincubated with 200 μM phosphotyrosine (Sigma) for 1 h. Likewise anti-RHAMM antibodies were preincubated with 200 μg RHAMM fusion protein before incubating with membranes as above.

Immunoprecipitation

Cells were stimulated with HA, and lysed as above. Each sample (500 μg of protein) was incubated with anti-p125^{FAK} mAb (4 μg/ml monoclonal anti-FAK, Transduction Laboratories and rabbit anti-mouse IgG (10 μg/ml; Sigma) for 1 h at 4°C by mixing end-over-end. To precipitate, 100 μl protein G-Agarose (GIBCO BRL) was added to each tube and the samples were mixed end-over-end for another 30 min at 4°C. The beads were pelleted by brief centrifugation at 13,000 rpm and washed three times with RIPA buffer. The proteins were released from the beads by boiling in Laemmli sample buffer then subjected to SDS-PAGE and anti-phosphotyrosine immunoblotting as above. To assess whether equal quantities of FAK were immunoprecipitated from control and treated samples, the blots were stripped and reprobed with purified anti-FAK (10 μg/ml antibody 2A7; a generous gift from J.T. Parsons, University of Virginia, Charlottesville, VA).

Immunofluorescent Staining

Vector control-*ras* 10T½, MR-C3-4D and MR-C3-5C fibroblasts were grown on untreated or fibronectin-coated glass coverslips for 24 h. The cells were exposed to HA (10 ng/ml), anti-RHAMM or control treatment for 1 min and 15 min. At the appropriate time periods, the media was aspirated, the cells rinsed with PBS, and then fixed with 3% paraformaldehyde (Sigma)/PBS for 10 min. Cells were washed three times for 10 min with wash solution (10% FCS/PBS containing 0.02% sodium azide), permeabilized with 0.2% Triton X-100/PBS for 5 min, and washed three more times. The fixed cells were incubated at 37°C for 1 h with anti-phosphotyrosine mAb (5 μg/ml; 4G10, UBI), anti-vinculin mAb (1:50; Sigma), or appropriate IgG controls (Sigma) in wash solution. After washing five times, coverslips were incubated with goat anti-mouse Cy3 (1:500; Jackson) for 3 h. After washing, the coverslips were mounted onto glass slides using Fluoromount (BDH). Observations and photomicrographs were obtained with a Zeiss Axiovert 35 M fluorescent microscope using epifluorescence.

Timelapse cinemicrography

To monitor random locomotion, 6 x 10^4 cells were plated in 25 cm^2 tissue culture flasks 24 hours prior to measurement. Cell locomotion was quantified using an IM 35 inverted microscope (Zeiss, Germany) to which a video camera (Hamamatsu CCD, Inc., Japan) was attached. The cells were maintained at 37°C using a heated platform (TRZ 3700, Zeiss, Germany). Motility was measured using image analysis (Image 1, Universal Imaging Corp., Westchester, PA, USA). This

program allows quantification of nuclear displacement in a sequence of digitilized images.

Flow cytometry

Cells were harvested using Hank's Balanced Salt Solution (HBSS) plus 20 mM Hepes, 0.05% sodium azide and 2.0 mM EDTA for 5 minutes, and were maintained at 4°C throughout the procedure. The cells were washed in the same solution except in the absence of EDTA, and then incubated with anti-peptide$^{aa268-288}$ antiserum (1:50 dilution; Hardwick *et al.*, 1992) for 30 minutes. Following this, the cells were incubated with fluoroscein-conjugated goat anti-rabbit antiserum (1:300 dilution, Sigma) for a further 30 minutes and were then fixed with 3% paraformaldehyde. The surface expression of RHAMM was studied using immunofluorescence flow cytometry. Normal IgG was used as a control at each timepoint analyzed.

HA binding assay

Proteins were electrophoresed on 10% SDS-PAGE gel and transblotted onto a nitrocellulose membrane in a Tris/glycine buffer containing 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.3 at 80 v for 1 h in a cold room. The membrane was blocked in 10 mM Tris-C1, pH 8.0, containing 150 mM NaC1 (TBS), 0.05% Tween 20) and 5% skim milk powder for 1 h at room temperature and then incubated with biotinylated HA (Yang *et al.*, 1994), diluted (1:2,000) in the same buffer overnight at 4°C. The membrane was washed with Tween-TBS extensively and then incubated with a streptavidin-peroxidase conjugate. Binding was visualized with

chemiluminescence according to kit instructions (ECL Kit, Amersham) that uses luminal and hydrogen peroxide to visualize the bound peroxidase.

Focus formation

Cells were grown to 50% confluence and, in the case of RHAMM antisense transfected clones, transfected with Ha-*ras* using lipofectin as above or in the case of RHAMM sense transfected clones, buffer only. The cultures were then grown for 3-4 weeks in DMEM medium containing 10-20% FBS. The medium was changed every 1-3 days for up to 4 weeks until foci formed. Foci were visualized with methylene blue staining and were counted/dish.

Growth in soft agar

5 x 10^3 cells/ml were plated in bactoagar (1.25%) containing αMEM 10% FBS for 7-10 days, as described (Egan *et al.*, supplemented, 1984). Colonies of cells were counted/plate. The plating efficiency of each cell line was similar (75%).

Subcutaneous tumor formation and tail vein assay

Cells were grown to confluence, washed with HBSS and released from the substratum in HBSS containing 2.5 mM EDTA. If cells did not release (i.e. 10T½ cells) they were scraped from the substratum with a rubber policeman. Exclusion of trypan blue indicated that >90% of cells treated in this manner were viable. C3 female mice were injected with 1-5 x 10^6 cells subcutaneously into the right hind leg and maintained for 3-6 weeks when tumors routinely become apparent. Animals were euthanized, tumors were removed, weighed and pieces processed for histology using paraffin embedding techniques. Lungs were also excised, examined for tumor

nodules which, when present, were also processed for paraffin sections.

For experimental metastasis assays (Egan *et al*,, 1989) 5 x 10^5 cells were injected into the tail vein. The mice were maintained for six weeks, euthanized, lungs removed and occurrence of tumor nodules assessed by processing tissue for histology and examining tissue sections for tumor nodules.

Acknowledgements:

This work was supported by an NCIC, MRC and Hyal Pharma. grants to E.A.T. C.H. was supported by an MRC Studentship, B.Y. by an MHRC Fellowship and S.S. by an NCIC Fellowship. A.H.G. is a Terry Fox Scientist of the NCIC and E.A.T. is a CHRF Scholar.

References

Alexander, C.M. and Werb, Z. (1992). Targeted disruption of the tissue inhibitor of metalloproteinases gene increases the invasive behaviour of primitive mesenchymal cells derived from embryonic stem cells *in vitro*. J. Cell Biol. *118*:727-739.

Bartolazzi, A., Peach, R., Aruffo, A. and Stamenkovic, I. (1994). Interaction between CD44 and hyaluronate is directly implicated in the regulation of tumor development. J. Exp. Med. *180*:53-66.

Behrend, E.J., Craig, A.M., Wilson, S.M., Denhardt, D.T. and Chambers, A.F. (1994). Reduced malignancy of *ras*-transformed N1H 3T3 cells expressing antisense osteopontin RNA. Cancer Res. *54*:832-837.

Borsi, L., Carnemolla, B., Nicolo, G., Spina, B., Tanara, G., Zardi, L. (1992). Expression of different tenascin isoforms in normal, hyperplastic and neoplastic human breast tissues. Int. J. Canc. *52*:688-692.

Brand, T., MacLellan, W.R., and Schneider, M.D. (1993). A dominant-negative receptor for type β transforming growth factors created by deletion of the kinase domain. J. Biol. Chem., *268*:11500-11503.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and Turner, C. (1988). Focal adhesions: trans-membrane junctions between the extracellular matrix and the cytoskeleton. Ann. Rev. Cell. Biol. *4*:487-525.

Calalb, M.B., Poste, T.R. and Hanks, S.K. (1994). Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for *src* family kinases. Mol. Cell. Biol. *15*:954-963.

Castle, V.P., Ou, X., O'Rourke, K. and Dixit, V.M. (1993). High level thrombospondin 1 expression in two NIH 3T3 cloned lines confers serum and anchorage-independent growth. J. Biol. Chem. *268*:2899-2903.

Chong, A.S.F., Boussy, I.A., Graf, L.H. and Scuderi, P. (1992). Stimulation of IFN-γ, TNFα and TNF-β secretion in IL-2 activated T cells: costimulatory roles for LFA-1, LFA-2, CD44, and CD45 molecules. Cell Immunol. *144*:69-79.

Damsky, C. and Webb, Z. (1992). Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. Curr. Opin. Cell Biol. *4*:772-781.

Egan, S.E., Wright, J.A., Jarolim, L., Yanagihara, K., Bassin, R.H. and Greenberg, A.H. 1987. Transformation by oncogenes encoding protein kinases induces the metastatic phenotype. Science *238*:202-205.

Entwistle, J., Zhang, S., Yang, B., Wong, C., Qun, L., Hall, C., A, J., Mowat, M., Greenberg, A.H. and Turley, E.A. (1995). Characterization of the murine gene encoding the hyaluronan receptor RHAMM. Gene, in press.

Evans, S.C., Lopez, L.C. and Shur, B.D. (1993). Dominant negative mutation in cell surface β1, 4-galactosyltransferase inhibits cell-cell and cell-matrix interactions. J. Cell Biol. *120*:1045-1057.

Fraser, R. and Laurent, T. (1993). Hyaluronate. FASEB J. *6*:2397-2404.

Giancotti, F.G. and Ruoslahti, E. (1990). Elevated levels of the α5β1 fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. Cell *60*:849-859.

Gunning, P., Leavitt, J., Muscat, G., Ng, S.Y. and Kedes, L. (1987) A human β actin expression vector system directs high-level accumulation of antisense transcripts. Proc. Natl. Acad. Sci. USA *84*:4831-4835.

Günthert, U. (1993). CD44:A multitude of isoforms with diverse functions. Curr. Topics in Microbiol. Immunol. *184*:47-63.

Günthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haubmann, I., Matzku, S., Wenzel, A., Ponta, H., Herrlich, P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell *65*:13-24.

Hall, C.L., Wang, C., Lange, L.A. and Turley, E.A. (1994). Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity. J. Cell Biol., in press.

Hardwick, C., Hoare, K., Owens, R., Hohn, H.P., Hook, M., Moore, D., Cripps, V., Austen, L., Nance, D.M. and Turley, E.A. (1992). Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. J. Cell Biol. *117*:1343-1350.

Hiro, D., Ito, A., Matsuta, K. and Mori, Y. (1986). Hyaluronic acid is an endogenous inducer of interleukin-1 production by human monocytes and rabbit macrophages. B.B.R.C., *140*:715-722.

Hoare, K., Savani, R.C., Wang, C., Yang, B. and Turley, E.A. (1993). Identification of hyaluronan binding proteins using a biotinylated hyaluronan probe. Conn. Tis. Res. *30*:117-126.

Howlett, A.R. and Bissell, M.J. (1993). The influence of tissue microenvironment (stroma and extracellular matrix) in the development and function of mammary epithelium. Epithel. Cell Biol. *2*:79-89.

Hynes, R.O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. Cell *69*:11-25.

Jones, P.C., Schmidhauser, C. and Bissell, M.J. (1993). Regulation of gene expression and cell function by extracellular matrix. Crit. Rev. Eukayot. Gene Expr. *3*:137-154.

Juliano, R.C. and Haskill, S. (1993). Signal transduction from the extracellular matrix. J. Cell Biol. *120*:577-585.

Kariko, K., Kuo, A., Boyd, D., Okada, S.S., Cines, D.B. and Barnathan, E.S. (1993). Overexpression of urokinase receptor increases invasion without altering cell

migration in human osteosarcoma cell line. Cancer Res. *53*:3109-3117.

Kashles, O., Yarden, Y., Fischer, R., Ullrich, A. and Schlessinger, J. (1991). A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by hetero dimerization. Mol. Cell Biol. *11*:1454-1463.

Khokha, R., Zimmer, M.J., Graham, C.H., Lala, P.K. and Waterhouse, P. (1992). Suppression of invasion by inducible expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in B16-F10 melanoma cells. J. Natl. Cancer Inst. *84*:1017-1022.

Klewes, L., Turley, E.A. and Prehm, P. (1993). The hyaluronate synthase from a eukaryotic cell line. Biochem. J. *290*:791-795.

Knudson, C.B., and Knudson, W. (1993). Hyaluronan-binding proteins in development, tissue homeostasis and disease. FASEB J., *7*:1233-1241.

Leibl, E.C. and Martin, G.S. (1992). Intracellular targetting of $pp60^{src}$ expression: localization of v-src to adhesion plagues is sufficient to transform chicken embryo fibroblasts. Oncogene 1992 *7*:2417-2428.

Lin, C.Q. and Bissell, M.J. (1993). Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J. *7*:735-743.

Lo, S.H. and Chen, L.B. (1994). Focal adhesion as a signal transduction organelle. Canc. Met. Rev. *13*:9-24.

Matsumoto, K., Matsumoto, K., Nakamura, T. and Kramer, R.H. (1994). Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase ($pFAK^{125}$) and promotes migration and invasion by oral squamous cell carcinoma cells. J. Biol. Chem. *269*:31807-31813.

McGlade, J., Brunkhorst, B., Anderson, D., Mbamalu, G., Settleman, J., Dedhar, S., Rozakis-Adcosk, M., Chen, L.B. and Pawson, T. (1993). The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. EMBO J. *12*:3073-3081.

Murphy-Ullrich, J.E. and Hook, M. (1989). Thrombospondin modulates focal adhesions in endothelial cells. J. Cell Biol. *109*:1309-1312.

Murphy-Ullrich, J.E., Lightner, V.A., Aukhil, I., Yan, Y.Z., Erickson, H.P., Hook, M., S.O. (1991). Focal adhesion integrity is downregulated by the alternatively spliced domain of human tenascin. J. Cell Biol., *115*:1127-1136.

Noble, P.W., Lake, F.R., Henson, P.M. and Riches, D.W.H. (1993). Hyaluronate activation of CD44 induces insulin-like growth factor expression by a tumor necrosis factor α dependent mechanism in murine macrophages. J. Clin. Invest. *91*:2368-2377.

Qian, F., Vaux, D.L. and Weissman, F.L. (1994). Expression of the integrin $\alpha_4\beta_1$ on melanoma cells can inhibit the invasive stage of metastasis formation. Cell *77*:335-347.

Ratner, S. (1992). Lymphocyte migration through extracellular matrix. Invasion Met. *12*:82-100.

Rodriguez-Fernández, J.L.R., Geiger, B., Salomon, D. and Ben-Ze'ev, A. (1993). Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility and anchorage-dependent growth of 3T3 cells. J. Cell Biol. 122:1285-1294.

Ruoslahti, E., Noble, N.A., Kagami, S. and Border, W.A. (1994). Integrins. Kidney Inter. *45*, Suppl. 44:S17-S22.

Saarialho-Kene, U.K., Kovacs, S.O., Pentland, A.P., Olevid, J.E., Welgus, H.G. and Parks, W.C. (1993). Cell-matrix interactions modulate interstitial collagenase expression by human keratinocyte actively involved in wound healing. J. Clin. Invest. *92*:2858-2866.

Samuel, S.K., Hurta, R.A.R., Spearman, M.A., Wright, J.A., Turley, E.A. and Greenberg, A.H. (1993). TGF-β_1 stimulation of cell locomotion utilizes the hyaluronan receptor RHAMM and hyaluronan. J. Cell Biol. *123*:749-758.

Schaller, M.D., Borgman, C.A., Cobb, B.S., Vines, R.R., Reynolds, A.B. and Parsons, J.T. (1992). $pp125^{FAK}$, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc. Natl. Acad. Sci. USA. *89*:5192-96.

Schaller, M.D. and Parsons, J.T. (1994). Focal adhesion kinase and associated proteins. Curr. Opin. Cell Biol. *6*:705-710.

Schlaepfer, D.D., Hanks, S.K., Hunter, T. and van der Geer, P. (1994) Nature *372*:786.

Schwartz, M.A. (1993). Signaling by integrins: implications for tumorigenesis. Cancer Res. *53*:1503-1506.

Shapiro, S., Doyle, G.A.D., Parks, W.C., Ley, T.J., and Welgus, H.G. (1993). Molecular mechanisms regulating the production of collagenase β TIMP in U937 cells: Evidence for involvement of delayed transcriptional activation and enhanced mRNA stability. Biochemistry *32*:4286-4292.

Stetler-Stevenson, W.G., Aznavoorian, S., Liotta, L.A. (1993). Tumor cell interactions with the extracellular matrix during invasion and metastasis. Amer. Rev. Cell Biol. *9*:541-573.

Sy, M.S., Guo, Y.J. and Stamenkovic, I. (1991). Distinct effects of two CD44 isoforms on tumor growth *in vivo*. J. Exp. Med. *174*:859-66.

Turley, E.A. (1992). Hyaluronan and cell locomotion. Cancer Met. Rev. *11*:21-30.

Turley, E.A. and Tretiak, M. (1984). Glycosominoglycans produced by murine melanoma variants *in vivo* and *in vitro*. Cancer Res. *45*:5098-5105.

Turley, E.A., Tretiak, M. and Tanguay, K. (1987). Effect of glycosaminoglycons and enzymes on the integrity of human placental amnion as a barrier to cell invasion. J.N.C.I. *78*:787-795.

Turley, E.A., Austin, L., Vandeligt, K. and Moore, D. (1991). Hyaluronan and a cell associated hyaluronan binding protein regulate the locomotion of *ras*-transformed cells. J. Cell Biol. *112*:1041-1047.

Varner, J.A., Fisher, M.H. and Juliano, R.L. (1992). Ectopic expression of integrin alpha 5/beta 1 suppresses *in vitro* growth and tumorigenicity of human colon carcinoma cells. Mol. Biol. Cell. *3*:232a.

Wehrle-Haller, B., and Chiquet, M. (1993). Dual function of tenascin: Simultaneous promotion of neurite growth and inhibition of glial migration. J. Cell Sci., *106*:597-610.

Werb, T., Tremble, P.M., Behrendtsen, E., Crowley, E. and Damsky, C.H. (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. *109*:877-889.

Woods, A. and Couchman, J.R. (1988). Focal adhesions and cell matrix interactions. Collagen Cell. Res. *8*:155-182.

Yang, B., Yang, B., Savani, R.C. and Turley, E.A. (1994). Identification of a common hyaluronan binding motif in the hyaluronan proteins RHAMM, CD44 and link protein. EMBO J. *13*:286-296.

Zachary, I. and Rozengurt, E. (1992). Focal adhesion kinase ($p125^{FAK}$): a point of convergence in the action of neuropeptides, integrins and oncogenes. Cell *71*:891-894.

TABLE 1: CHARACTERISTICS OF RHAMM TRANSFECTED 10T½ CLONES.

Cell Line	Densitometry of RHAMM Western Analysis	FACS* Mean Fluorescent Intensity	Rate of Motility (μM/hr ± SEM) Control	Rate of Motility (μM/hr ± SEM) Anti-RHAMM Antibody	Foci/Dish	Nuclear Overlap Ratio	Subcut. tumors in mice	Spont. Mets.
a) 10T½ fibroblast	0.31	2.4	10.0 ± 0.85	8.6 ± 1.2	13 ± 4	0.01	0/4	0/8
C3 fibrosarcoma	1.35	85.0	35.1 ± 2.2	10.2 ± 1.4	77 ± 9	0.45	4/4	N.D.
b) Genomic RHAMM Transfectants								
Vector only	0.54	2.5	10.6 ± 0.9	9.9 ± 0.4	12 ± 3	0.02	0/4	0/8
λ4-2 - 10T½	1.29	82.5	36.6 ± 4.4	N.D.	N.D.	0.36	N.D.	N.D.
λ4-6 - 10T½	2.20	43.0	31.8 ± 2.0	N.D.	N.D.	0.25	N.D.	N.D.
λ4-10 - 10T½	1.08	74.8	25.6 ± 1.7	10.9 ± 1.7	150 ± 7	0.49	4/4	N.D.
λ4-12 - 10T½	2.77	116.0	36.1 ± 2.2	11.7 ± 0.5	249 ± 16	0.42	4/4	4/8+
c) RHAMM cDNA Transfectants								
Vector only	0.35	3.9	9.8 ± 0.5	9.8	11 ± 4	0.01	0/4	N.D.
cDNA 5-10T½	2.65	74.8	24.5 ± 2.3	10.1 ± 2.1	240 ± 10	0.41	4/4	N.D.
cDNA-3T3	N.D.	N.D.	N.D.	N.D.	250 ± 10	N.D.	4/4	N.D.
MR10T½-4	1.56	81.3	11.1 ± 0.6	9.3 ± 1.2	0	0.01	0/4	N.D.

*Corrected for background fluorescence in the presence of normal IgG_1 ; ND: Not determined; 10T½ cells were transfected with either genomic RHAMM (λ4) for clones 2, 6, 10 and 1
RHAMM cDNA (RHAMM 1v4 Entwistle *et al*, submitted) for clone cDNA-5 or RHAMM cDNA mutated in its HA binding domains clone MR10T½--4,; NIH-3T3 cells were transfecte
with RHAMM cDNA (RHAMM 1v4) for clone cDNA-3T3. Characteristics were determined as outlined in methods. Motility rate was obtained by tracking 100 cells.
+ mean of 5 colonies/lung

Table 2
SUPPRESSION OF TRANSFORMATION BY EXPRESSION OF RHAMM MUTATED IN ITS HA BINDING DOMAIN

Cell Line*	Growth in Soft Agar Colonies ± S.E.	Nuclear Overlap Ratio	Foci/Dish	Motility Rate μM/hr ± S.E.
10T½	0	0.01	0	9.5 ± 1.3
C3-Vector Control	77 ± 9	0.47	>60	27.5 ± 1.2
MR-C3-4D	0	0.005	0	3.5 ± 0.8
MR-C3-5B	1 ± 1	0.004	0	1.8 ± 0.5
MR-C3-5C	0	0.005	0	2.0 ± 0.6

*The H-*ras* transformed C3 fibrosarcoma was transfected either with vector control (C3-vector control) or with RHAMM (Entwistle *et al.*, submitted) mutated in its HA-binding domain (MR-C3-4D, MR-C3-5B, MR-C3-5C), while 10T½ is the non-transformed parental line of the C3.

Figure 1. Reversion of H-*ras* transformed C3 fibrosarcoma by expression of RHAMM mutated in its HA binding domain. A) Strategy for mutating the hyaluronan binding domains (boxed) of RHAMM. Lysines (K) and arginines (R) were altered as indicated and this alterations has been shown to destroy the hyaluronan binding properties of the RHAMM protein (Yang *et al.*, 1994). RHAMM fusion protein was prepared from the intact cDNA (designated RHAMM fusion protein), electrophoresed and assayed for biotinylated HA binding in a transblot assay. Fusion protein produced from the RHAMM cDNA that was mutated as described in Methods. Mutated RHAMM fusion protein did not bind hyaluronan. B) C3 cell lysates were prepared from cells that were transfected with vector control (lane 1 and 5), or mutated RHAMM cDNA (lanes 2 to 4 and 6 to 8 which represent clones MRC3-4D, 5B, 5C) were electrophoresed and analyzed for RHAMM using a Western transblot analysis (lane 1 to 4). The blot was then stripped and reprobed with biotinylated HA (lanes 5 to 8). RHAMM was overexpressed in the transfected cell lines but RHAMM from these cells bound less hyaluronan than controls consistent with a dominant negative action by the mutated protein. C) Total RHAMM expression on the cell membrane assessed by FACS revealed major increases in MRC3 clones relative to the vector control. D) Cell lysates were obtained from C3-vector controls (lane 1), 10T½ fibroblasts (lane 2) and the three MRC3 clones, 4D,-5B,-5C (lanes 3 to 5 respectively), electrophoresed then $p21^{ras}$ was visualized in a Western transblot analysis using a pan-specific anti-*ras* antibody. All of the MRC3 transfected clones expressed high levels of $p21^{ras}$ comparable to C3 (lane 1).

Figure 1

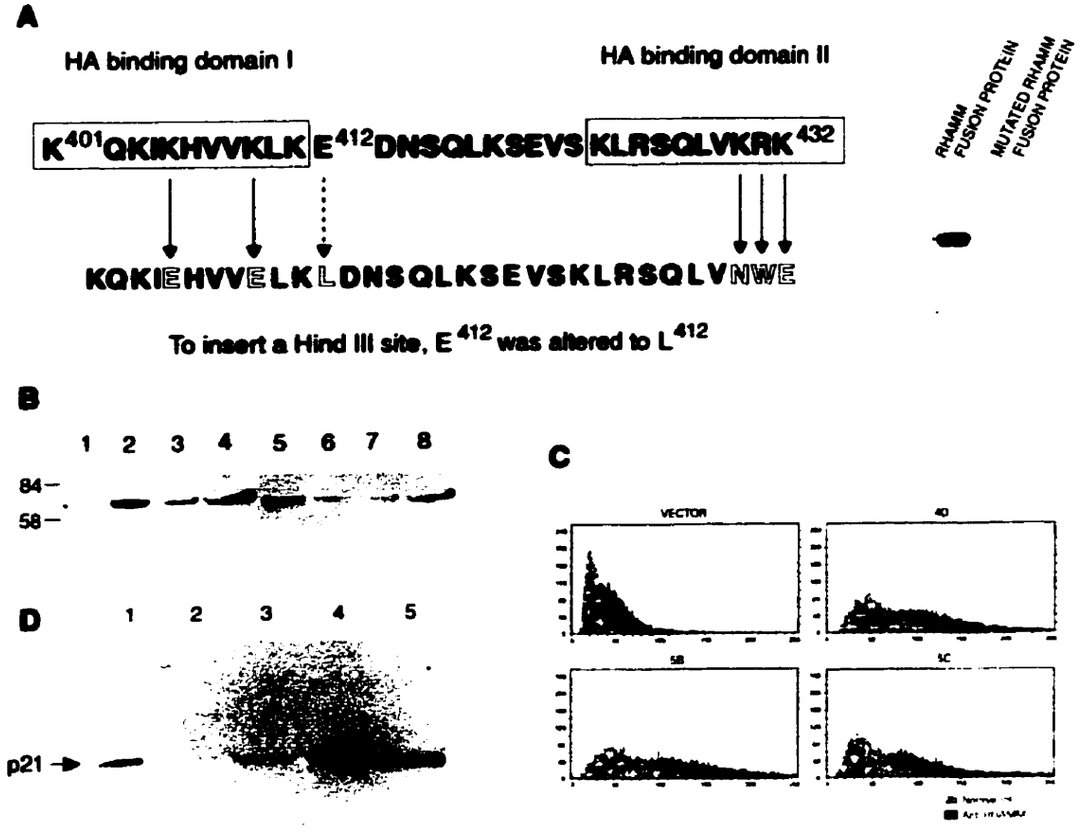

A
HA binding domain I
HA binding domain II
K401QKIKHVVKLK E412DNSQLKSEVS KLRSQLVKRK 432
RHAMM FUSION PROTEIN
MUTATED RHAMM FUSION PROTEIN
KQKIEHVVELKLDNSQLKSEVSKLRSQLVNWE
To insert a Hind III site, E 412 was altered to L 412
B
1 2 3 4 5 6 7 8
84—
58—
C
VECTOR
40
5B
5C
D
1 2 3 4 5
p21

Figure 2. Morphological and growth properties of dominant negative mutant transfected C3 cells. (A-C) Cell morphology of transfected cell lines including 10T½ parent line. (A), vector transfected C3 cells (B) and MR-C3 transfected cells (C). The MRC3 cells resemble the non-transformed 10T½ line appearing flattened and contact inhibited in contrast to the vector transfected *ras*-transformed parent C3 line. D) Growth curve of vector control transfected C3 cells (□), MRC3-3D (♦), MRC3-5B (□), and MRC3-5C (◊) clones. 5 x 10^4 were plated and the number of cells was counted each day with a Coulter counter. The vector control transfected cells grow more rapidly than the mutant RHAMM transfected *ras* - transformed cells and reach a much higher saturation density. E) A focus forming assay was performed as described in Methods using vector control transfected C3 cells and MRC3-4D clone. The vector controls formed multiple foci in dense culture while the cells transfected with mutated RHAMM did not form foci. F) Mice injected with either C3 parent line (1) or vector control (2) formed large fibrosarcoma (arrows) by 3 weeks after injection. Mice injected with MRC3-4D (3) or 5C (4) clones did not form tumors after six months observation. G) The vector control C3 control injected into the tail vein of mice formed multiple lung colonies in the mouse as shown in the upper panel. All three of the MRC3 clones (4D, 5B and 5C) did not form detectable colonies (bottom panel).

Figure 2

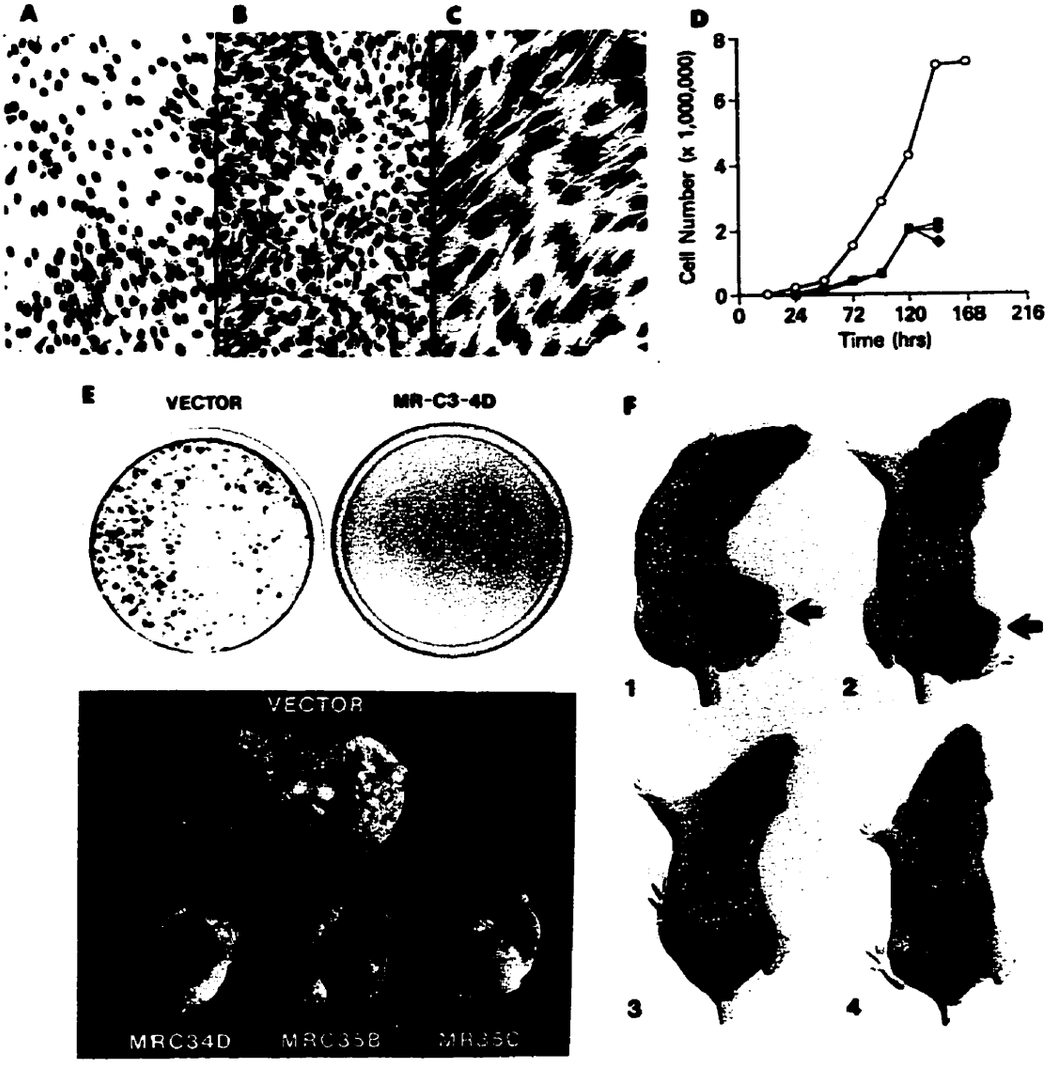

A
B
C
D
Cell Number (x 1,000,000)
0
2
4
6
8
0
24
72
120
168
216
Time (hrs)
E
VECTOR
MR-C3-4D
VECTOR
MRC34D
MRC35B
MR35C
F
1
2
3
4

Figure 3. Overexpression of dominant suppressor RHAMM prevents tyrosine phosphorylation, focal adhesion kinase dephosphorylation and focal adhesion turnover in *ras*-transformed fibroblasts. A) Twenty-four hour cultures of vector control-C3 fibroblasts (left) and MR-C3-4D (right) were stimulated with HA (10 ng/ml) for 0 (lane 1), 1 min (lane 2), 5 min (lane 3) or 15 min (lane 4) prior to cell lysis. Equal protein concentrations from each sample were run on SDS-PAGE gels, transferred to nitrocellulose, and subjected to anti-phosphotyrosine immunoblot analysis. Vector control cells displayed a rapid and transient increase in the tyrosine phosphorylation of p185, p125, p115, and p85 (arrows) followed by a decrease in the phosphorylation of these protein bands, as did the *ras*-10T½ C3 fibroblasts (not shown). The level of tyrosine phosphorylation in the MR-C3-4D cells did not change with HA treatment. Similar results were obtained with the clone MR-C3-5C (not shown). The molecular markers (M) are 190, 125, 88 and 65 kDa. B) Cell cultures of vector control-C3 fibroblasts and mutated RHAMM overexpressers MR-C3-4D were stimulated as above, for 1 min (lanes 2, 5) or 15 min (lanes 3, 6) with HA or with buffer alone (lanes 1, 4), then lysed in RIPA buffer. Immunoprecipitation was performed with anti-FAK (lanes 1-6) or mouse IgG control (lane 7) followed by SDS-PAGE and anti-phosphotyrosine immunoblot analysis. Vector control fibroblasts (lanes 1-3) exhibit an increase followed by a decrease in FAK phosphorylation. The MR-C3-4D cells (lanes 4-6) show no change in FAK phosphorylation with HA treatment. C) Immunofluorescent localization of vinculin and phosphotyrosine in vector control-C3 and MR-C3-4D fibroblasts before and after

HA treatment. Fibroblasts were incubated in the absence (0) or presence of 10 ng/ml HA for 1 min (1, IgG) or 15 min (15) before fixation and staining with anti-vinculin (a, b), anti-phosphotyrosine (c, d), or mouse IgG. Vector control cells (a, c) exposed to HA for 1 min show an increase in both focal adhesions (anti-vinculin) and anti-phosphotyrosine staining that then decreases by 15 min of treatment. MR-C3-4D (b, d) and MR-C3-5C (not shown) display focal adhesions and phosphotyrosine staining that do not change with treatment.

e [illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

Figure 3

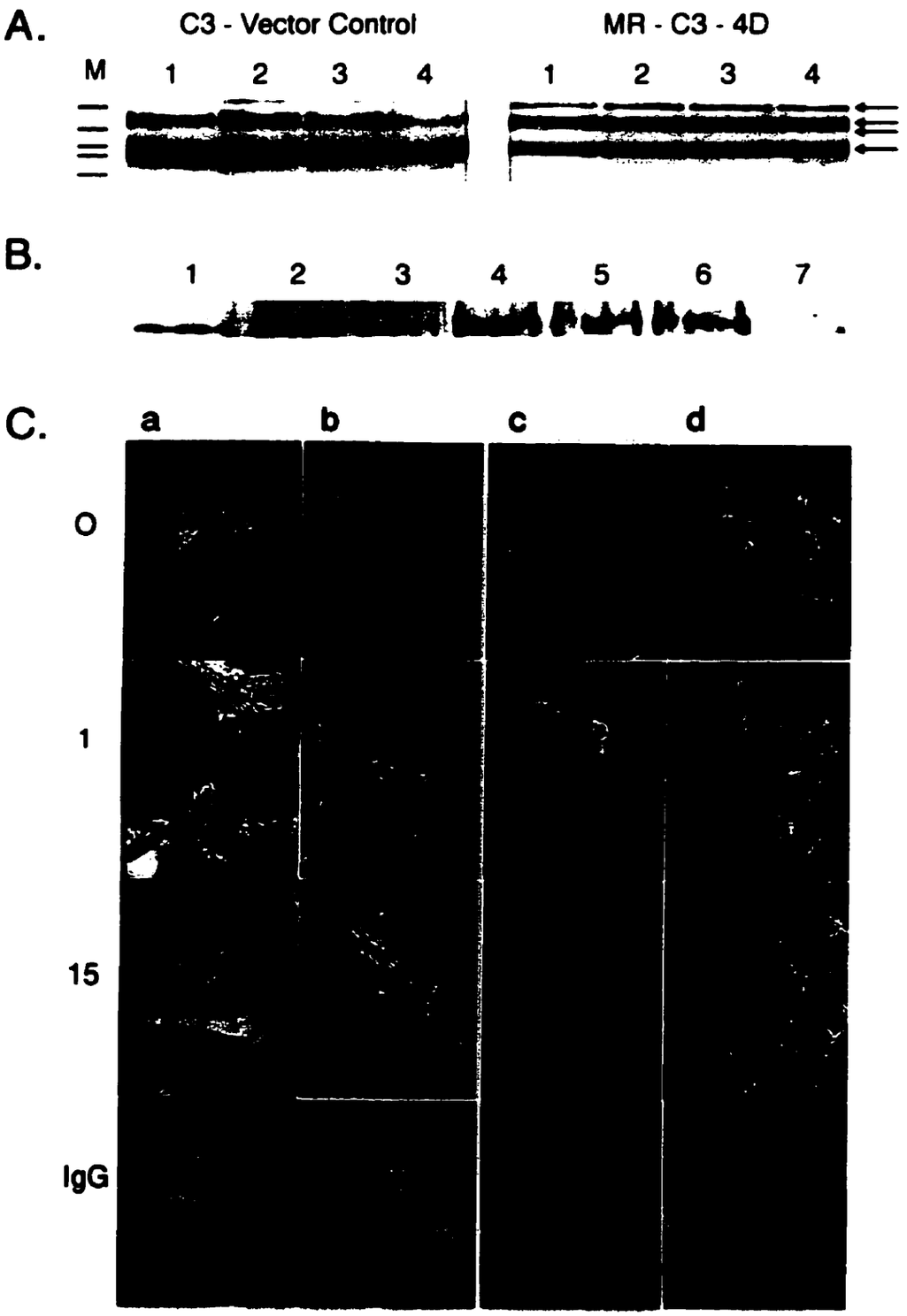
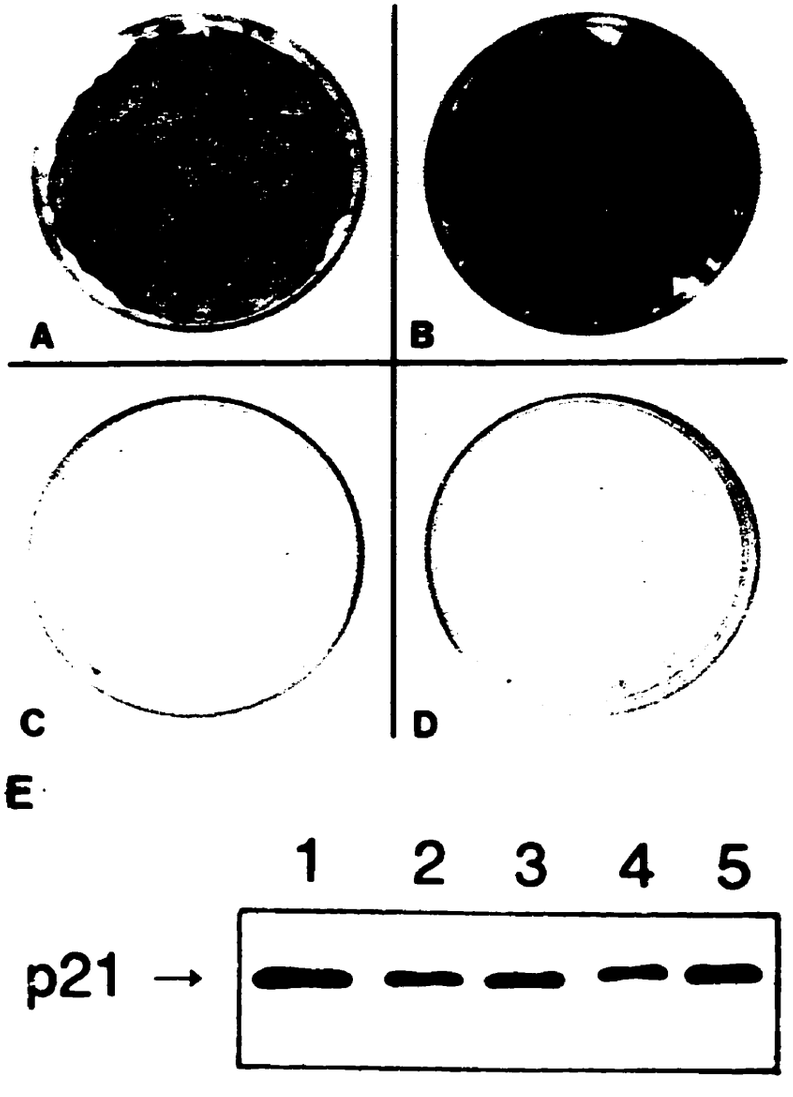

A.
C3 - Vector Control
MR - C3 - 4D
M
1
2
3
4
1
2
3
4
B.
1
2
3
4
5
6
7
C.
a
b
c
d
0
1
15
IgG

Figure 4. Inhibition of transformation by mutant H-*ras* in fibroblasts expressing antisense RHAMM clones OR1 and OR2; A) 10T½ parent cells transfected with *ras*; B) Transfection of vector control 10T½ cells with *ras;* C) OR1 clone transfected with *ras;* and D) OR2 clone transfected with *ras*. *Ras* vector yielded exhibited multiple large foci formed in controls but, clones transfected with RHAMM antisense did not form foci. E) *Ras* protein is expressed in *ras*-transfected OR1, OR2 fibroblasts. All cells were transfected with *ras* (*ras*-transformed C3 cells lane 1, antisense vector only 10T½ clone 1, lane 2; antisense vector only 10T½ clone 2, lane 3, OR1-cells lane 4, OR2-cells lane 5) expressed p21 *ras* protein as detected in a Western assay with a pan-*ras* antibody. Levels of expression (lane 2-5) are approximately equivalent and are higher than normally expressed in parental 10T½ cells (see Figure 2D).

Figure 4



Chapter 3

$pp60^{c\text{-}src}$ is Required for Cell Locomotion Regulated by the Hyaluronan Receptor RHAMM

C.L. Hall, L.A. Lange, D.A. Prober, S. Zhang and E.A Turley

Oncogene, Volume 13, 1996, pages 2213-2224

Preface

In the previous chapter, it was shown that the overexpression of RHAMM not only results in elevated cell locomotion, but is transforming and bestows tumorigenic and metastatic capabilities upon murine fibroblasts cell lines. In addition, it was illustrated that RHAMM is required for transformation by oncogenic *ras*. Indeed, the loss of functional RHAMM in *ras*-transformed fibroblasts inhibits cell locomotion, triggers a reversion in phenotype to that typical of non-motile, non-transformed fibroblasts and impedes signaling with focal adhesions (Chapter 2, Hall *et al.*, 1995). With these results in mind, we proposed that RHAMM acts downstream of *ras* to regulate signal transduction events within focal adhesions, and that these signals then contribute to transformation. In support of this hypothesis, a number of reports suggest the signaling within focal adhesions is critical for the regulation of growth

and cell motility (see Zachary & Rozengurt, 1992; Lo & Chen, 1994; Clark & Brugge, 1995). Focal adhesions harbour key regulatory proteins, among them protein tyrosine kinases such as FAK and src family kinases. Because RHAMM mediated cell locomotion requires protein tyrosine phosphorylation (Chapter 1, Hall *et al.*, 1994), it is possible that RHAMM induced tyrosine phosphorylation within focal adhesions is also a key event in RHAMM-, and hence *ras*-, induced transformation. Thus, HA:RHAMM generated tyrosine phosphorylation signals could influence cytoskeletal and adhesion structures to permit elevated cell motility, and could regulate downstream events leading to alterations in gene expression and cell proliferation. As a result, in this chapter, we wish to identify a key tyrosine kinase required for RHAMM signaling within focal adhesions and to determine whether the identified kinase is required for cell locomotion or transformation, or both.

In this chapter we examine the hypothesis that the proto-oncogene tyrosine kinase $pp60^{c\text{-}src}$ acts as a key downstream component of the HA:RHAMM signaling pathway. Src was examined for involvement in the HA:RHAMM signaling pathway for the following reasons: **1)** Preliminary data indicated that src and RHAMM co-precipitated and co-localized in locomoting chick heart fibroblasts (Turley, 1989; unpublished data). **2)** Both RHAMM (Turley *et al.*, 1990; X. Yang and E. Turley, unpublished data) and src (see Resh, 1990; Clark *et al.*, 1994; Clark & Brugge, 1995, and references therein) are associated with the cytoskeleton and focal adhesions. **3)** Src associates with, and can phosphorylate, FAK (see Richardson & Parsons, 1995 and references therein), a substrate in the RHAMM signaling pathway (Hall *et al.*,

1994 & 1995). **4)** Herbimycin A, a potent inhibitor of src, inhibits HA:RHAMM induced cell motility (Hall *et al.*, 1994). **5)** Cytoskeleton- and adhesion-associated src protein can form complexes with *ras* regulatory proteins, providing a possible mechanism for RHAMM regulation of *ras*-transformation (Schlaepfer *et al.*, 1994; Chang *et al.*, 1995). **6)** Src is involved in growth factor induced mitogenesis (Luttrell *et al.*, 1988; Wilson *et al.*, 1989; Twamley-Stein *et al.*, 1993; Roche *et al.*, 1995) and hence, is a candidate for RHAMM induced mitogenesis. The primary goal of this chapter is to determine the role of src in RHAMM mediated cell motility and focal adhesion turnover. The secondary goal of this chapter is to make observations, if any, regarding the role of src in RHAMM induced tumorigenesis, creating the groundwork for future directions. The final goal of this chapter is to create a model exhibiting how RHAMM, src and *ras* function together to control such distinct processes as cell motility and cell proliferation.

Specific Objectives

1. To determine role of src in fibroblast cell locomotion by examining the motility rates of fibroblasts lacking $pp60^{c\text{-}src}$, src (-/-) fibroblasts. To examine how the expression src, and mutants of src, in these src (-/-) fibroblasts effects cell motility. To define the involvement of RHAMM in src (-/-) and wild-type fibroblast cell locomotion.
2. To determine the role of src in RHAMM induced cell locomotion by a) expressing dominant negative src in *RHAMM1v4*-transformed fibroblasts and

b) inhibiting src function in *ras*-transformed fibroblasts.

3. To determine if activated src, i.e. v-src, can stimulate elevated cell locomotion, even when RHAMM function is inhibited.
4. To determine the role of src in RHAMM induced focal adhesion turnover by a) examining focal adhesions in *RHAMM1v4*-transformed fibroblasts expressing dominant negative src and b) examining focal adhesions in v-*src*-transformed 10T½ and U21 (anti-sense RHAMM-10T½).
5. To examine the physical association between RHAMM and src in *ras*-transformed fibroblasts which express RHAMM1v4.
6. To determine if src kinase activity, or another domain(s) of src, is required for RHAMM mediated cell locomotion. To demonstrate if src becomes activated after HA stimulation in *ras*-transformed fibroblasts.

Contribution

The contribution of the above authors to this work, is as follows. All of the data presented is a result of experiments carried out by myself (CLH) and in some cases with the technical assistance of LAL and DAP. In addition, the transfections of 10T½ and U21 fibroblasts were done by SZ. The compilation of data, the research of materials and background literature and the writing of the manuscript were conducted by CLH. All work and writing were conducted with the guidance of EAT.

References

Chang J-H, Gill S, Settleman J and Parson SJ. (1995). c-Src regulates the simultaneous rearrangement of actin cytoskeleton, p190RhoGAP, and p120RasGAP following epidermal growth factor stimulation. *J. Cell Biol.*, **130**, 355-368.

Clark EA and Brugge JS. (1995). Integrins and signal transduction pathways: the road taken. *Science*, **268**, 233-239.

Clark EA, Sanford SJ and Brugge JS. (1994). Regulation of protein tyrosine kinases in platelets. *Trends Biochem. Sci.*, **19**, 464-469.

Hall CL, Wang C, Lange LA and Turley EA. (1994). Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity. *J. Cell Biol.*, **126**, 575-588.

Hall CL, Yang B, Yang X, Zhang S, Turley M, Samuel S, Lange LA, Wang C, Curpen GD, Savani RC, Greenberg AH and Turley EA. (1995). Overexpression of the hyaluronan receptor RHAMM is transforming and is also required for H-*ras* transformation. *Cell*, **82**, 19-28.

Lo SH and Chen LB. (1994). Focal adhesion as a signal transduction organelle. *Cancer Metastasis Rev.*, **13**, 9-24.

Luttrell DK, Luttrell LM and Parsons SJ. (1988). Augmented responsiveness to epidermal growth factor in murine fibroblasts that overexpress $pp60^{c\text{-}src}$. *Mol. Cell. Biol.*, **8**, 497-501.

Resh MD. (1990). Membrane interactions of pp60$^{v\text{-}src}$: a model for myristylated tyrosine protein kinases. *Oncogene*, **5**, 1437-1444.

Richardson A and Parsons JT. (1995). Signal transduction through integrins: a central role for focal adhesion kinase? *BioEssays*, **17**, 229-236.

Roche S, Koegl M, Barone CA, Roussel MF and Courtneidge SA.(1995). DNA synthesis induced by some but not all growth factors requires src family protein tyrosine kinases. *Proc. Natl. Acad. Sci.*, **89**, 5192-5196.

Schlaepfer DD, Hanks SK, Hunter T and van der Geer P. (1994). Integrin-mediated signal transduction linked to ras pathway by GRB2 binding to focal adhesion kinase. *Nature*, **372**, 786-791.

Turley EA. (1989). The role of a cell-associated hyaluronan-binding protein in fibroblast behaviour. *In* Biology of Hyaluronan. *Ciba Found. Symp.*, **143**, 121-137.

Turley EA, Brassel P and Moore D. (1990). A hyaluronan binding protein shows a partial and temporally regulated codistribution with actin on locomoting chick heart fibroblasts. *Exp. Cell Res.*, **187**, 243-249.

Twamley-Stein GM, Pepperkok R, Sansorge W and SA Courtneidge. (1993). The src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. *Proc. Natl. Acad. Sci.*, **90**, 7696-7700.

Wilson LK, Luttrell DK, Parsons JT and Parsons SJ. (1989). pp60$^{c\text{-}src}$ tyrosine kinase, myristylation, and modulatory domains are required for enhanced mitogenic

responsiveness to epidermal growth factor seen in cells overexpressing c-*src*. *Mol. Cell. Biol.*, **9**, 1536-1544.

Zachary I and Rozengurt E. (1992). Focal adhesion kinase ($p125^{FAK}$): a point of convergence in the action of neuropeptides, integrins, and oncogene. *Cell*, **71**, 891-894.

$pp60^{c\text{-}src}$ is Required for Cell Locomotion Regulated by the Hyaluronan Receptor RHAMM

C.L. Hall, L.A. Lange, D.A. Prober, S. Zhang and E.A. Turley

Manitoba Institute of Cell Biology and Departments of Pediatrics and Physiology, University of Manitoba, 100 Olivia Street, Winnipeg, Manitoba, Canada R3E 0V9

Correspondence to: Dr. Eva Turley

Tel: (204) 787-2137

Fax: (204) 787-2190

E-mail: eturley@cc.umanitoba.ca

Running Title: **Role for c-src in RHAMM induced cell locomotion**

Key words: migration, focal adhesions, cytoskeleton, tyrosine phosphorylation

Abstract

The tyrosine kinase $pp60^{c\text{-}src}$ has been implicated as a regulator of focal adhesion formation and cell spreading. Here we show that c-src also regulates cell motility and is a key component in the signaling pathway triggered by the motogenic hyaluronan receptor RHAMM, which has been shown to regulate focal adhesion turnover and to regulate *ras*. Fibroblasts derived from mice lacking src, [src (-/-)], have a random locomotion rate that is significantly slower than the corresponding wild-type fibroblasts. Cell locomotion in these mutant cells is restored by the expression of c-src containing a functional kinase domain, but not by the expression of a kinase-deficient src or by a truncated src containing only functional SH2 and SH3 domains. RHAMM is also required for the restoration of src (-/-) cell locomotion. Thus, the motility of cells expressing c-src is reduced to src (-/-) levels by anti-RHAMM blocking antibodies while the cell locomotion of src (-/-) fibroblasts remains unaffected by anti-RHAMM antibodies. We predict that src acts downstream of RHAMM in the regulation of motility, since the expression of a dominant negative src significantly inhibits RHAMM-dependent, *ras* and serum regulated cell locomotion, the expression of v-src enhances cell motility in a RHAMM independent fashion, and there is a physical and functional association between src and RHAMM in *ras*-transformed cells. However, we suggest that RHAMM regulates focal adhesion turnover via additional src-independent mechanisms. Thus, v-src is unable to turnover focal adhesions in the absence of RHAMM. These results directly demonstrate for the first time a role for src in the

regulation of cell locomotion and confirm a key and complex role for src in the regulation of the actin cycle.

Introduction

Cell locomotion is a fundamental requirement for embryogenesis, wound healing, tissue remodelling, the migration of immune cells during inflammatory responses and the metastasis of malignant tumor cells. During these events, a concert of growth factors, motility factors and extracellular matrix (ECM) components direct the crawling of cells via signaling pathways that impinge upon the cytoskeletal architecture and adhesive interactions of a cell (see Stoker & Gherardi, 1991; McCarthy & Turley, 1993; Stossel, 1993; Huttenlocher *et al.*, 1995; Lauffenburger & Horwitz, 1996). One aspect of cell locomotion in particular, the formation of cell-substratum adhesions, requires a tyrosine phosphorylation signal that is initiated upon attachment and is necessary for cell spreading and migration (see Burridge *et al.*, 1992; Lo & Chen, 1994; Richardson & Parsons, 1995; Lauffenburger & Horwitz, 1996). The phosphorylation of focal adhesion proteins in part, stimulates the formation of focal adhesion complexes and most likely results from the activation of the focal adhesion kinase, FAK, and the associated src family kinases, fyn, csk and src.

The proto-oncogene tyrosine kinase $pp60^{c\text{-}src}$ has been implicated as a modulator of cytoskeletal organization, adhesion and migration. Both c-src and its viral counterpart, v-src, have been localized to the cytoskeleton, cell-substratum

adhesive structures and cell-cell junctions (see Resh, 1990; Clark *et al.*, 1994; Clark & Brugge, 1995, and references therein; Cartwright *et al.*, 1993; Oude Weernink & Rijksen, 1995) where they can influence the alterations in cell-cell and cell-matrix adhesion that promote cell dissociation, cell spreading and migration (David-Pfeuty & Singer, 1980; Tarone *et al.*, 1985; Holme *et al.*, 1986; Kellie *et al.*, 1986; Marchisio *et al.*, 1987; Warren & Nelson, 1987; Kellie, 1988; Vallés *et al.*, 1990; Nermut *et al.*, 1991; Matsuyoshi *et al.*, 1992; Behrens *et al.*, 1993; Hamaguchi *et al.*, 1993; Nakamura *et al.*, 1993; Rodier *et al.*, 1995; Takeda *et al.*, 1995) via tyrosine phosphorylation of cytoskeletal and adhesion proteins (see Kellie *et al.*, 1991; Wu & Parsons, 1993; Chang *et al.*, 1995; Takeda *et al.*, 1995 and references therein). Src has been demonstrated to act as a regulator of cytoskeletal reorganization and adhesion during neurite outgrowth (Maness & Cox, 1992; Ignelzi *et al.*, 1994), regulated secretion (Oddie *et al.*, 1989; Ely *et al.*, 1994), bone remodelling (Soriano *et al.*, 1991; Boyce *et al.*, 1992), cell spreading (Kaplan *et al.*, 1995), cell dissociation (Rodier *et al.*, 1995) and bacterial invasion (Dehio *et al.*, 1995). The association of src with endothelial cell migration (Bell *et al.*, 1992), carcinoma cell scattering (Rodier *et al.*, 1995) and fibroblasts cell spreading (Kaplan *et al.*, 1995) strongly suggest that src plays a critical role in cell motility.

The involvement of src in adhesion, cytoskeletal organization and migration may be dependent upon its cellular localization, its modular structure and its state of activation. Src is composed of a myristylated and unique N-terminal region, SH2 and SH3 domains, a kinase domain and a regulatory C-terminal region (see Cooper &

Howell, 1993). This domain structure contributes to the regulation of src which occurs via intramolecular binding of the SH2 domain to a phosphorylated tyrosine (Y527) in the regulatory C-terminal domain (MacAuley & Cooper, 1989). Src must be in an active configuration to bind to the cytoskeleton since a stable association between src and cytoskeletal components depends upon tyrosine phosphorylation and is mediated by the SH2 domain of src (Fukui *et al.*, 1991; Okamura & Resh, 1994). However, both the SH2 and SH3 domains of src are required for association with focal adhesion protein complexes (Kaplan *et al.*, 1994). In these complexes, src co-associates with FAK via its SH2 domain (Cobb *et al.*, 1994; Schaller *et al.*, 1994) and paxillin via SH3 binding (Weng *et al.*, 1993). Here src can come into close proximity with other cytoskeletal proteins, ECM receptors and regulators of signaling pathways (see Clark & Brugge, 1995). These interactions place src in an ideal position to modulate pathways important for adhesion and migration.

To further understand the role for src in cell locomotion, we have examined cells that utilize the hyaluronan (HA) receptor RHAMM for elevated cell locomotion. Hyaluronan is a ubiquitous component of the ECM and has been shown to promote cell motility, adhesion and proliferation during morphogenesis, wound repair, inflammation and metastasis (Laurent & Fraser, 1986 & 1992; Toole *et al.*, 1989; Turley, 1992; Sherman *et al.*, 1994; Savani & Turley, 1995). These effects of HA are mediated via cell surface receptors, namely CD44 (Aruffo *et al.*, 1990) and RHAMM (Hardwick *et al.*, 1992; Entwistle *et al.*, 1995). In particular, RHAMM has emerged as a critical regulator of adhesion and migration in a variety of cell types

including fibroblasts, lymphocytes, smooth muscle cells and epithelial cells locomoting in response to the *ras* oncogene, injury or growth factors (see Turley, 1992; Hall & Turley, 1995; Savani & Turley, 1995; Entwistle *et al.*, 1996). These results place RHAMM as a central player in cell responses to motogenic stimuli. In fibroblasts, the overexpression of the RHAMM isoform, RHAMM1v4, is sufficient to induce elevated cell locomotion, decreased adhesion, increased growth, transformation and metastatic potential (Hall *et al.*, 1995). Furthermore, RHAMM1v4 is required to signal motility in *ras*-transformed cells (Hall *et al.*, 1995) suggesting that this receptor acts downstream of ras. Cell lines that exhibit a RHAMM locomotory pathway require tyrosine phosphorylation and focal adhesion turnover, events that coincide with transient phosphorylation of focal adhesion kinase (FAK) (Turley *et al.*, 1991; Hall *et al.*, 1994; Hall *et al.*, 1995). It is likely that in this pathway focal adhesion structures are formed for the purpose of signal transduction and that the subsequent exchange of traditional focal adhesions for smaller podosome-like adhesions is necessary for an increased motility rate.

Here, we show that src kinase is required for elevated cell locomotion in fibroblasts; that v-src can induce cell motility in 10T½ fibroblasts in a RHAMM independent fashion; that dominant negative src inhibits RHAMM mediated cell locomotion; and that there is a physical and functional association between src and RHAMM in *ras*-transformed fibroblasts. This evidence suggests that src acts downstream of RHAMM to signal cell locomotion and further implicates src as a regulator of the actin cycle.

Results

Src kinase and the hyaluronan receptor RHAMM are required for random fibroblast motility

To determine the role for src in cell motility, we examined the random locomotion rates of both wild-type fibroblasts and src (-/-) fibroblasts, isolated from mice with a disrupted src gene (Soriano *et al.*, 1991). The src (-/-) fibroblasts had a significantly ($p < 0.001$) lower basal motility rate than did the corresponding wild-type cells. Reintroduction of c-src into the src (-/-) cells restored cell locomotion to the level of wild-type fibroblasts (Figure 1A). The addition of anti-RHAMM blocking antibodies inhibited cell locomotion in cells expressing c-src, but had no significant effect on src (-/-) cell motility, despite the fact that both cell lines express RHAMM (Figure 1B; Hall & Turley, 1995). In these mouse cells and under the culture conditions described in methods, two specific RHAMM isoforms were expressed. It is likely that the major band is RHAMM1v4, the isoform shown to regulate motility, since Northern analysis using exon 4 as a probe showed this isoform to be equally expressed in wild type and mutant cells (data not shown). The origin and the significance of the second smaller isoforms is not known at present.

We next examined the role of the domains of src in locomotion. Expression of either c-src or activated srcA, a Y527F mutation that prevents negative regulation of the kinase, induced an approximately 60% increase in the motility of src (-/-) cells (Figure 1C). Cells expressing srcAM, which is made kinase deficient by a K295M mutation in the ATP binding region of the kinase domain but is in an open

configuration due to the Y527F mutation (Kaplan *et al.*, 1994), did not exhibit elevated motility. As well, the expression of a truncated src containing the unique N-terminal region, as well as SH2 and SH3 domains (src251; Kaplan *et al.*, 1994) resulted in only a small, but significant ($p < 0.05$), 15% increase in cell motility. Thus, these data indicate that only in the presence of functional src kinase and RHAMM is a large increase in cell motility observed. In addition, while expression of both the srcAM and src251 mutants are sufficient to promote more rapid cell spreading and focal adhesion formation in these src (-/-) cells (Kaplan *et al.*, 1995), induction of elevated cell motility requires a functional src kinase domain.

We next assessed whether elevated cell motility induced by the overexpression of RHAMM requires src kinase. Fibroblasts expressing the RHAMM isoform RHAMM1v4 exhibit a significant increase in cell motility that is dependent on RHAMM and HA production (Entwistle *et al.*, 1995; Hall *et al.*, 1995). To ascertain whether RHAMM requires src to signal motility, we introduced a dominant negative src into these RHAMM1v4-overexpressing 10T½ fibroblasts. SrcAM, described above, is kinase defective but the binding sites for target proteins, SH2 and SH3 domains, are intact thus allowing this protein to act as a dominant negative. Expression of srcAM in *RHAMM1v4*-transformed fibroblasts resulted in a significant decrease in cell motility (Figure 2A) to a level comparable to parental 10T½ fibroblast motility (see Figure 4A). Thus, a dominant negative src ablates the effect of RHAMM1v4 overexpression. Interestingly, inactivation of src did not alter the adhesion structures or the actin cytoskeleton in RHAMM1v4 transfected cells, which

remained typical of motile cells (Figure 2B).

Src specific reagents block RHAMM induced cell locomotion in ras-transformed cells

To determine if src is required for HA:RHAMM promoted locomotion in *ras*-transformed cells, we analyzed the effect of src specific agents on *ras*-transformed cell locomotion. Previously, the microinjection, electroporation or electro-transjection of anti-src antibodies have been shown to interfere with src function in neurite outgrowth (Kremer *et al.*, 1991), growth factor-induced mitogenesis (Twamley-Stein *et al.*, 1993), angiotensin II signaling (Marrero *et al.*, 1995) or the activation of phospholipase C in platelets (Dhar & Shukla, 1994). Likewise, we have previously shown that the microinjection of src antibodies, a src peptide found to be an inhibitor of src (peptide 1, VAPSDSIQAEEWYFGKITRRE; Sato *et al.*, 1990) or a substrate peptide of src family kinases (peptide 2, KVRKIGEGTYGVVKK; UBI) dramatically inhibits HA:RHAMM induced cell locomotion (Hall & Turley, 1995). However, experiments were difficult to interpret since we also found that the control injections alone would often inhibit baseline motility by up to 50%. As another approach to this question, we have covalently linked the above peptides to Penetratin 1 internalization vector (Appligene; Derossi *et al.*, 1994) which provides an alternative technique for the introduction of peptides into cells. In these experiments, cells were serum starved to reduce the rate of locomotion and to reduce the endogenous production of HA. Cells can then be stimulated to move by the addition of HA or stimulatory anti-RHAMM antibody. By this method neither peptide had

an effect on baseline locomotion, but both prevented an HA induced increase in cell motility (Figure 3). These results are consistent with a downstream requirement for src in a RHAMM regulated motility pathway in *ras*-transformed fibroblasts.

v-Src induces cell locomotion independently of RHAMM

We have reasoned that since the above results suggest that src acts downstream of RHAMM, an activated src (i.e. v-src) may promote elevated cell locomotion even in the absence of RHAMM function. Transformation of 10T½ fibroblasts with v-*src* results in a similar increase in the baseline cell motility (Figure 4A) to that obtained with mutant active *ras* (Trahey *et al.*, 1987; Partin *et al.*, 1988; Ocheing *et al.*, 1991; Turley *et al.*, 1991; Hall *et al.*, 1995). Anti-RHAMM blocking antibodies which inhibit locomotion in normal (Figure 4A) and *ras*-transformed fibroblast (Turley *et al.*, 1991; Hardwick *et al.*, 1992), had no effect on the cell locomotion of v-*src*-transformed fibroblasts, demonstrating, that in contrast to *ras*-transformed cells, RHAMM is not required for the high motility rates observed in v-*src*-transformed cells. These results show that activated src can compensate for RHAMM by elevating cell motility and provide further evidence that src acts downstream of RHAMM to signal cell locomotion. Interestingly, however, v-src was unable to signal focal adhesion turnover in cells that express little RHAMM due to antisense transfection (Fig. 4B). v-*src* transformation into 10T½ cells generally reduced actin stress from formation. In the absence of RHAMM, however, stress fibres remained well developed (Fig. 4B). These results indicate that src alone cannot compensate for this effect of RHAMM and that additional signals must therefore be generated from

RHAMM signaling focal adhesion turnover.

Role of src in ras transformed cells: RHAMM co-localizes and co-precipitates with src

We have previously shown that *ras*-transformed fibroblasts overexpress the RHAMM1v4 isoform and require RHAMM to achieve their high rate of cell locomotion (Turley *et al.*, 1991; Hardwick *et al.*, 1992; Hall *et al.*, 1994 & 1995). In these fibroblasts, RHAMM and ras localize to the cell membrane on ruffling surfaces and in extending lamellae, a pattern consistent with the cellular protrusions utilized for cell motility (Turley & Auersperg, 1989; Turley *et al.*, 1990; Turley, 1992). As these cells also require tyrosine kinase activity (Hall *et al.*, 1994) and src function (see above) for RHAMM induced cell locomotion, we assessed the relationship between RHAMM and src in these fibroblasts. Immunofluorescent localization of RHAMM with a polyclonal anti-RHAMM antibody displays variable staining within cellular processes and along the edge and at the base of the lamellae (Figure 5A). Co-immunofluorescence with a monoclonal anti-src antibody to the unique N-terminal region (anti-src 2-17) reveals variable patterns of src/RHAMM co-localization most often occurring at the cell periphery and in regions the cell body corresponding to endosomes (David-Pfeuty & Nouvian-Dooghe, 1990; Kaplan *et al.*, 1992). Several examples of the patterns of staining for these proteins and the extent of their co-distribution are detailed in Fig. 5A. A similar co-distribution was observed in cells overexpressing RHAMM1v4 (data not shown).

We next assessed whether src and RHAMM co-immunoprecipitate in *ras*-

transformed fibroblasts. Under non-detergent or weak detergent conditions, such as digitonin or CHAPS buffers (Figure 5B), RHAMM is detected in anti-src immunoprecipitations. In these *ras* transformed cells, RHAMM occurred predominantly as a 125 kDa protein, unlike the mouse fibroblasts (Fig. 1B) and unlike previous reports using different cell lines (Hardwick *et al.*, 1992). The reason for this discrepancy in molecular weights is unknown but may represent differential glycosylation (Hardwick *et al.*, 1992) or alternate splicing (Entwistle *et al.*, 1995). Both RHAMM and src immunoprecipitation are blocked by the addition of src peptide to the immune complexes (data not shown) and antibody binding to RHAMM in the immunoblot assay is prevented by pre-incubation with RHAMM peptide (Figure 5B). We were unable to detect src in RHAMM immunoprecipitates and only 2% of the cellular RHAMM co-precipitates with src. We also were unable to detect RHAMM in anti-fyn or anti-yes immunoprecipitates from these cells. As both RHAMM (Klewes *et al.*, submitted) and src (see Resh, 1990 & 1994) are associated with the plasma membrane via glycosylphosphatidylinositol and lipid anchors respectively, it is likely that these proteins cluster together in the glycolipid-enriched membrane regions or associate indirectly through integral docking proteins (see Rudd *et al.*, 1993; Anderson, 1994). The association of src family kinases and GPI-linked proteins in insoluble or easily disrupted membrane aggregates (Sargiacomo *et al.*, 1993; Rodgers *et al.*, 1994; Gorodinsky & Harris, 1995) may account for the low level of co-immunoprecipitation we observe here, in spite of the strong co-distribution detected by immunofluorescent localization.

Src becomes activated in ras-transformed 10T1/2 fibroblasts during RHAMM signaling

Cell lines overexpressing RHAMM1v4, and exhibiting increased cell motility, had elevated src activity relative to the parental cell line, 10T½, which has low levels of RHAMM1v4 and a slower motility rate in growth media (Figure 6A; Hall *et al.*, 1995). Furthermore, *ras*-transformed fibroblasts grown in defined media for a minimum of 12 h, respond to HA or stimulatory anti-RHAMM with an inducible increase in cell locomotion (1.4-1.9 fold; Hall *et al.*, 1994). Addition of HA (data not shown) or stimulatory anti-RHAMM antibody to these cells also resulted in a transient increase in src kinase activity as observed by src immunoprecipitation auto-kinase assays (Figure 6B) or by a src peptide kinase assay (1.4-1.7 fold increase in kinase activity). This rapid activation followed by a drop in kinase activity resembles the time course for RHAMM induced tyrosine phosphorylation and focal adhesion turnover (Hall *et al.*, 1994).

Discussion

The events linking the extracellular ligand:cell surface receptor signaling to cytoskeletal rearrangements and motility signals are incompletely understood. The association of the protein tyrosine kinase $pp60^{c\text{-}src}$ with cytoskeletal and adhesion structures, the existence of numerous cytoskeletal substrates for src and the involvement of src in cytoskeletal reorganization (see Introduction), all suggest that src is a regulator of the cellular architecture. Indeed, the recent identification of a

role for src in growth factor induced cytoskeletal arc formation (Chang *et al.*, 1995), epithelial scattering (Rodier *et al.*, 1995) and integrin mediated cell spreading (Kaplan *et al.*, 1995) suggest that src is a critical component of pathways regulating cytoskeletal assembly. Here we show that src regulates random cell motility in fibroblasts, is a component of the cell motility pathway triggered by the extracellular ligand, hyaluronan, and one of its motogenic cell surface receptors RHAMM, and is a co-requirement, together with RHAMM, for cytoskeletal disassembly.

Protein tyrosine phosphorylation is a critical event in the formation of new focal adhesions and hence is required for cell spreading. In particular, src has recently been illustrated to play an important role in focal adhesion formation and the initiation of cell spreading in fibroblasts (Kaplan *et al.*, 1995). These authors found that cells expressing src initiate focal adhesion assembly and cell spreading on fibronectin more rapidly than src (-/-) fibroblasts. However, when cells were examined after the initial spreading period, there were no apparent differences in the focal adhesions of src (-/-) cells and those containing src (Bockholt *et al.*, 1993; Kaplan *et al.*, 1995). These results suggest that c-src functions in the early stages of focal adhesion formation and are consistent with a role we have assigned to src in cell motility which likely requires the turnover of these structures. The SH2 and SH3 domains of src appear to be sufficient to positively regulate cell spreading (Kaplan *et al.*, 1995), but we present direct evidence that a functional src kinase domain is required to induce locomotion (Figure 1) suggesting that src kinase regulates additional signaling events to promote cell motility.

Previous studies indicate that src in carcinoma cells is associated with enhanced cell scattering in response to EGF and FGF-1 (Rodier *et al.*, 1995); src activity is associated in endothelial cells with enhanced migration (Bell *et al.*, 1992); and src-family proteins are associated with chemotaxis in leukocytes induced by cytokines (Ryan *et al.*, 1995) and N-formyl peptides (Ptasznik *et al.*, 1995). As well, a role for src in cell motility is consistent with the physiological role for src in osteoclast function (Boyce *et al.*, 1992) and neurite outgrowth (Ignelzi *et al.*, 1994). Cell movement is an essential component of bone remodelling and the ruffled borders formed for bone resorption in osteoclasts resemble membrane structures in motile cells. The cytoskeletal and adhesive changes that occur during bone resorption and neurite extension are very similar to those events required for motility and suggest that src may have similar functions in numerous cell types. Nevertheless, src may not always have a positive effect on cell motility. In 3T3 fibroblasts, v-src expression has been reported to have no effect on random cell locomotion and inhibit chemotaxis in response to PDGF-BB (Kundra *et al.*, 1994) or in some cases to actually decrease cell migration (Sankar *et al.*, 1995). Further, migration in response to collagen does not require src (Rodier *et al.*, 1995). Thus the effect of src on cell motility may depend on the cell type, growth conditions, stimulus and differentiation state of the cell.

RHAMM is a glycophosphatidyl inositol (GPI)-linked cell surface receptor (Klewes *et al.*, submitted) that co-localizes with src in the ruffling membranes and podosomal regions of locomoting *ras*-transformed fibroblasts and weakly co-

associates with src in cell extracts. Similar to the association between other GPI-anchored cell surface receptors and src family kinases (see Rudd et al., 1993; Casey, 1995), RHAMM/src interactions are likely to occur within the membrane invaginations known as caveolae (Anderson, 1994; Klewes *et al.*, submitted). RHAMM activation triggers a transient increase in src kinase activity, perhaps by means of receptor clustering within caveolae. The unique amino-terminal region of src, which interacts with acidic phospholipids of the cell membrane (Resh, 1994), are likely important for these interactions as the microinjection of anti-src antibodies to this region inhibit RHAMM mediated cell locomotion (Hall & Turley, 1995). It has been proposed that GPI linked proteins may interact with intracellular proteins via lipid based mechanisms and such interactions may link RHAMM and src. Alternatively, RHAMM may interact with an integral docking protein that collects signaling complexes to its cytoplasmic domain.

We have previously shown that *ras* regulates both the expression of RHAMM and the production of HA, producing an autocrine-loop to stimulate elevated cell locomotion (Turley *et al.*, 1991; Hardwick *et al.*, 1992). Thus, in *ras*-transformed cells RHAMM is an absolute requirement for transformation, cell locomotion, focal adhesion turnover and the net dephosphorylation of FAK (Hall *et al.*, 1995). In addition, we now show that src kinase is required for RHAMM induced cell motility in *ras*-transformed cells. Based on our data and others (Chang *et al.*, 1995; Thomas *et al.*, 1995) we present one model in Figure 7 depicting the contribution of HA/RHAMM/src to cell movement via the control of the actin cycle. The continuous

gelation and reassembly of actin filaments and associated protein complexes are fundamental requirements for membrane protrusion and the formation of new adhesions at the front of the cell and the retraction/release at the trailing end. The precise balance of the actin cycle by a number of signaling cascades is intimately connected to, if not instructive to, cell locomotion. We suggest that HA interacts with RHAMM to promote src activity and src, in turn and in part, directly or indirectly influences actin fiber formation/gelation, membrane anchoring/release and the initial formation of cell-matrix adhesions. For example, src can affect the cytoskeleton through phosphorylation of substrates such as cortactin, which promote the anchoring of actin filaments into the plasma membrane (Okamura & Resh, 1995) or tensin which may influence aggregation of actin filaments at focal adhesions (Lo & Chen, 1994; Thomas *et al.*, 1995). Furthermore, at sites of adhesion a transient tyrosine phosphorylation of FAK, followed by dephosphorylation and focal adhesion turnover, is consistent with src activity (Fincham *et al.*, 1995) and elevated cell motility (Matsumoto *et al.*, 1994). It is apparent that in addition to src kinase, other signals from RHAMM or ras contribute to focal adhesion turnover and it is possible that these pathways converge at different points. Nevertheless, src is in an ideal position to influence the small GTP binding proteins, molecular switches shown to regulate lamellar extension and adhesion (Nobes & Hall, 1995). Further experimentation is required to identify the precise binding partners linking this pathway to effectors of motility, to identify other pathways involved and to determine the precise nature of the cytoskeleton in cell locomotion. Clearly other pathways

involving for instance phospholipase C, phosphatidylinositol-3 kinase, protein kinase C and small GTP protein switches play a critical and possibly variable role not only in the control of the actin cycle and cell locomotion, discussed here, but in pathways regulating cell proliferation.

The relationship between src and *ras* in signaling motility is not addressed in this study. Active mutant *ras* has previously been linked to up-regulated motility in several cell types and we have shown that this requires the presence of a functional RHAMM (Hall *et al.*, 1995). Previous studies have placed src upstream of ras in the regulation of transformation. Our studies on motility place src downstream of RHAMM and therefore likely downstream of ras. Since the signaling of motility and proliferation appear to be separately regulated, it is possible that src may regulate motility via a different relationship with ras than events controlled by this protein kinase that impact on proliferation. Studies are currently being conducted to assess the relationship of src and ras to each drug in signaling motility.

Materials and Methods

Cell Lines and Cell Culture Conditions

The cell lines derived from the established 10T½ murine fibroblast cell line, H-*ras*-10T½ CIRAS-3 (C3), and *RHAMM1v4*-10T½ (LR21; cDNA 5-10T½) and a 10T½ cell line expressing RHAMM anti-sense (U21) have previously been described (Egan *et al.*, 1987a; Hall *et al.*, 1995). These cells were maintained at 37°C in 5% CO_2 in a growth media of alpha-MEM (Gibco BRL) supplemented with 10% FBS

alternative (Fetal Clone III; Hyclone Laboratories). Wild-type and src (-/-) cell lines were kind gifts from K. Burridge (University of North Carolina, Chapel Hill) and P. Soriano (Fred Hutchinson Cancer Research Center, Seattle). These cells were derived from the spontaneous immortalization of mouse embryo fibroblasts from wild-type mice and mice lacking c-src due to a targeted disruption of the src gene (Soriano *et al.*, 1991; Thomas *et al.*, 1993) and maintained in high glucose DMEM containing 10% FBS (Intergen). The cells were never allowed to reach 100% confluency and every 3 days were subcultured using a trypsin/EDTA solution (0.25% Difco Bactotrypsin, 2 mM EDTA in PBS: 2.7 mM KCl; 1.1 mM KH_2PO_4; 138 mM NaCl; 8.1 mM Na_2HPO_4; pH 7.4). All experiments were performed at 24-32 h after subculture. For studies in defined media, cells were allowed to recover in the appropriate growth media for 12 h after passage, the cells were rinsed with HBSS (Gibco BRL) and fresh serum-free media containing 4.0 μg/ml transferrin (human) and 2.0 μl/ml insulin (bovine) was added. Cell were maintained in defined media for 12-18 h before further analysis.

Retroviral transfection and constructs

v-S*rc*-10T½ and v-*src*-U21 cells were obtained through v-*src*/neo retroviral infection of 10T½ and U21 cell lines as described previously (Egan *et al.*, 1987b). Transformed colonies were selected in media containing 0.6 mg/ml G418 and v-src expression was confirmed by immunoblot analysis. Src (-/-) or *RHAMM1v4*-10T½ cells were infected with retrovirus containing a hygromycin B selectable marker and the src constructs (Kaplan *et al.*, 1994): c-Src, SrcA (Y527F), SrcAM (K295M,

Y527F), or Src251 (truncated after 251 amino acids) kindly provided by K.B. Kaplan (Massachusetts Institute of Technology, Cambridge) and H.E. Varmus (NIH, Bethesda). Infected cells were selected in appropriate media containing 125 μg/ml hygromycin B (Sigma Chemical Company). For each of these constructs, isolated colonies were pooled and a heterogeneous population of cells expressing the appropriate construct was further analyzed.

Cell locomotion analysis

Random cell locomotion was quantified using a computerized time-lapse image analysis system (Image-1, Universal Imaging Corporation) attached to an Axiovert 100 or an IM 35 inverted microscope (Zeiss) via a video camera (Hammatsu CCD). In each experiment, the average cell motility of approximately 25-30 cells for 10T½-derived cell lines or 20-25 cells for src (-/-) derived cells was obtained by recording in 15 min intervals for a 2 h period. As cell motility may vary by up to 50% depending on the passage number, time after subculture, supplementary serum type and level of confluency, these factors were kept as constant as possible for each experiment and, as a result, different experiments may not be directly comparably. For all experiments, cells were plated to be 50% confluent, or to contain 25-30 cell/field at the time of time-lapse recording. HA (10 ng/ml), stimulatory anti-RHAMM (1:100,000; Hall *et al.*, 1994), monoclonal (3T3-5, 1:100) and rabbit polyclonal (anti-peptide 2, R3.4, 1:500; Hardwick *et al.*, 1992 or R10.1 against a RHAMM fusion protein, 1:500; Yang *et al.*, 1993) RHAMM blocking antibodies, Penetratin-coupled peptides (final concentration of 160 nM) and appropriate controls

were added to cell cultures 10-15 minutes prior to beginning locomotion analysis. For Penetratin coupled peptides, Peptide 1, CVAPSDSIQAEEWYFGKITRRE, and peptide 2, CKVRKIGEGTYGVVKK, were coupled to activated Penetratin according to manufacturer's instructions (Appligene, Oncor) and checked on an SDS-PAGE gel.

Immunofluorescent Staining

Fibroblasts grown for 24 h on sterile untreated or fibronectin coated (Bio-coat; VWR) coverslips in 6-well culture dishes in growth media were fixed as previously described (Hall *et al.*, 1994). For RHAMM and src co-localization, fixed cells were incubated with primary antibodies, polyclonal rabbit anti-RHAMM (R10.1, 1:100) and monoclonal mouse anti-src 2-17 (1:2000; Quality Biotech) or appropriate IgG controls, for 1 h at 37°C or overnight at 4°C. After washing, conjugated secondary antibodies, goat anti-mouse FITC (1:300; Sigma) and goat anti-rabbit TRITC (1:300; Sigma) were added for 1 h at room temperature. For focal adhesion and polymerized actin localization, fixed cells were incubated with monoclonal anti-vinculin (1:200; Sigma) for 1 h at 37°C followed by goat anti-mouse Cy3 (1:300; Jackson ImmunoResearch Laboratories) for 1 h at room temperature or with phalloidin conjugated to TRITC (1:500; Sigma) for 2 h at room temperature. Coverslips were mounted onto glass slides using fluoromount (BDH Inc.). Observations and photomicrographs were obtained with a Zeiss Axiovert 35M fluorescent microscope and a Contax 139 Quartz camera.

Immunoprecipitation

Twenty-four hour, 50% confluent cultures of H-*ras*-10T½ fibroblasts (C3) in growth

media were used for digitonin or CHAPS extraction. For each extraction, cells were washed 2 times with ice cold PBS followed by addition of either digitonin buffer (1% digitonin: 150 mM NaCl; 50 mM HEPES, pH 7.5; 1 mM Na_3VO_4; 1 mM EDTA; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 1 mM PMSF; Samelson *et al.*, 1990) or CHAPS buffer (10 mM CHAPS; 20 mM Tris-HCl, pH 8.3; 150 mM NaCl; 200 μM Na_3VO_4; 2 mM EDTA; 50 mM NaF; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 1 mM PMSF; Neet and Hunter, 1995) for 15-20 minutes on ice. Extract was gathered by gentle aspiration with a Pasteur pipette, transferred to a microcentrifuge tube and centrifuged on a benchtop microcentrifuge at high speed for 15 min at 4°C. The supernatant was collected and used for immunoprecipitation. For best results fresh supernatants were used. For each immunoprecipitation, cell extracts (400-500 μg protein) were incubated with anti-src 2-17 (2 μg/ml), mAb 327 (2 μg/ml; v-src Ab-1, Oncogene Science) or mouse IgG (2 μg/ml) or for mock immunoprecipitations, antibodies were incubated with lysis buffer alone, for 1 h at 4°C on a Nutator rotator (Becton Dickinson). To precipitate, 100 μl recombinant Protein G Agarose (GIBCO BRL) was added to each tube and the extracts were mixed for another 30 min at 4°C. The beads were pelleted by brief centrifugation and washed 3 times with extraction buffer. Proteins were released from beads by boiling in SDS-PAGE sample buffer (1 M Tris-HCl, pH 6.8; 2 % glycerol; 20% SDS; 1% β-mercaptoethanol; 0.1 % bromophenol blue) then subjected to SDS-PAGE and anti-RHAMM immunoblot analysis (see below).

Cell lysis and Immunoblot analysis

For immunoblot analysis of total cell lysates, 24 h cultures of fibroblasts in growth media were washed on ice with PBS and lysed by addition of ice cold RIPA buffer (25 mM Tris-HCl, pH 7.2; 0.1% SDS; 1% Triton X-100; 1% sodium deoxycholate; 0.15 M NaCl; 1 mM EDTA; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 1 mM PMSF). Lysates were scraped into microcentrifuge tubes after 10 min and were centrifuged at high speed for 15 min at 4°C. Protein concentrations of supernatents and BSA standards were determined using the *DC* protein assay (Bio-Rad Laboratories) and 10 μg of protein from each sample were separated by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes and subjected to anti-RHAMM immunoblot analysis. Additional protein binding sites on the nitrocellulose membrane were blocked by incubation with 5% defatted milk in TBS (50 mM Tris-HCl, pH 7.4; 200 mM NaCl) for 1 h at room temperature, followed by incubation with anti-RHAMM (R10.1, 1:500; or anti-peptide I, 1:500; Hardwick *et al.*, 1992) or anti-RHAMM peptide I pre-incubated with peptide (peptide 1; Hardwick *et al.*, 1992) in a 1% milk/TBS solution for 2 h at room temperature. Membranes were washed with 0.05% Tween 20/TBS (TTBS) 4 x 10 min and then incubated for 1 h at room temperature with goat anti-rabbit secondary antibody conjugated to HRP (1:5000 in 1% milk in TTBS). After washing as above, antibody binding was visualized by chemiluminescence according to manufacturer's instructions (ECL; Amersham Corp.).

Kinase Assays

Peptide kinase assays were performed on src immunoprecipitates from 10T½, H-*ras*-10T½, and *RHAMM1v4*-10T½. Immunoprecipitates were performed as above except RIPA buffer (see above) was used to obtain cell extracts and to wash the immunoprecipitations. Immunoprecipitated samples were either subjected to SDS-PAGE and src immunoblot analysis to check for equal amounts of precipitated src or assessed for kinase activity using the src family protein tyrosine kinase assay kit (Upstate Biotechnology Inc; UBI). For the src autokinase assay (O'Connor *et al.*, 1992), 24 h cultures of H-*ras*-10T½ in defined media were treated with HA (10 ng/ml) for the time periods indicated before lysis with RIPA buffer. Src immunoprecipitations from each timed sample were then suspendend in 30 µl of kinase buffer (20 mM Tris-HCl, pH 7.2; 5 mM $MnCl_2$) and incubated with ^{32}P-γ-ATP for 10 min before stopping the reaction with SDS-PAGE sample buffer and boiling samples for 3 min. Immunoprecipitated src samples were subjected to SDS-PAGE, gels were dried on gel dryer (Bio-Rad) and analyzed by autoradiography or by Phosphoimager (Molecular Dynamics) for src autophosphorylation and phosphorylation of immunoglobulin.

Acknowledgements

We gratefully acknowledge Dr. Ken Kaplan for providing us with the src constructs used in these studies and Drs. P. Soriano, Keith Burridge and Susanne Bockholt for proving us with the src (-/-) and wild-type fibroblasts. This work was supported by

Medical Research Council of Canada (MRC) to EAT and an MRC studentship to CLH.

References

Anderson RGW. (1994). *Semin. Immunol.*, **6**, 89-95.

Aruffo A, Stamenkovic I, Melnick M, Underhill CB and Seed B. (1990). *Cell*, **61**, 1303-1313.

Behrens J, Vakaat L, Friis R, Winterhager E, Van Roy F, Mariel MM and Birchmeier W. (1993). *J. Cell Biol.*, **120**, 757-766.

Bell L, Luthringer DJ, Madri JA and Warren. (1992). *J. Clin. Invest.*, **89**, 315-320.

Bockholt SM, Soriano P and Burridge K. (1993). *Mol. Biol. Cell*, **4**Suppl, abs.no 2097.

Boyce BF, Yoneda T, Lowe C, Soriano P and Mundy GR. (1992). *J. Clin. Invest.*, **90**, 1622-1627.

Burridge K, Turner CE and Romer LH. (1992). *J. Cell Biol.*, **119**, 893-903.

Cartwright CA, Mamajiwalla S, Skolnick SA, Eckhart W and Burgess DR. (1993). *Oncogene*, **8**, 1033-1039.

Casey PJ. (1995). *Science*, **268**, 221-225.

Chang J-H, Gill S, Settleman J and Parsons SJ. (1995). *J. Cell Biol.*, **130**, 355-368.

Clark EA and Brugge JS. (1995). *Science*, **268**, 233-239.

Clark EA, Shattil SJ, and Brugge JS. (1994). *Trends Biochem. Sci.*, **19**, 464-469.

Cobb BS, Schaller MD, Horng-Leu Z and Parsons JT. (1994). *Mol. Cell. Biol.*, **14**,

147-155.

Cooper JA and Howell B. (1993). *Cell*, **73**, 1051-1054.

David-Pfeuty T and Singer SJ. (1980). *Proc. Natl. Acad. Sci.*, **77**, 6687-6691.

Dehio C, Prévost M-C and Sansonetti PJ. (1995). *EMBO J.*, **14**, 2471-2482.

Derossi D, Joliot AH, Chassaing G and Prochiantz A. (1994). *J Biol Chem.*, **269**, 10444-10450.

Dhar A and Shukla SD. (1994). *J. Biol. Chem.*, **269**, 9123-9127.

Egan SE, Wright JA, Jarolim L, Yanaguhara K, Bassin RH and Greenberg AH. (1987b). *Science*, **238**, 202-205.

Ely CM, Tomiak WM, Allen CM, Thomas L, Thomas G and Parsons SJ. (1994). *J. Neurochem.*, **62**, 923-933.

Entwistle J, Hall CL and Turley EA. (1996). *J. Cell Biochem.*, **61**,569-577.

Entwistle J, Zhang S, Yang B, Wong C, Li Q, Hall CL, A J, Mowat M, Greenberg AH and Turley EA. (1995). *Gene*, **163**, 233-238.

Fukui Y, O'Brien MC and Hanafusa H. (1991). *Mol. Cell. Biol.*, **11**, 1207-1213.

Gorodinsky A and Harris DA. (1995). *J. Cell Biol.*, **129**, 619-627.

Hall CL and Turley EA. (1995). *J. Neuro-Oncol.*, **26**, 221-229.

Hall CL, Wang C, Lange LA and Turley EA. (1994). *J. Cell Biol.*, **126**, 575-588.

Hall CL, Yang B, Yang X, Zhang S, Turley M, Samuel S, Lange LA, Wang C, Curpen GD, Savani RC, Greenberg AH and Turley EA. (1995). *Cell*, **82**, 19-28.

Hamaguchi M, Matsuyoshi N, Ohnishi Y, Gotoh B, Takeichi M and Nagai Y.

(1993). *EMBO J.*, **12**, 307-314.

Hardwick C, Hoare K, Owens R, Hohn HP, Hook M, Moore D, Cripps V, Austen L, Nance DM and Turley EA. (1992). *J. Cell Biol.*, **117**, 1343-1350.

Holme TC, Kellie S, Wyke JA and Crawford N. (1986). *Br. J. Cancer*, **53**, 465-476.

Huttenlocher A, Sanborg RR and Horwitz AF. (1995). *Curr. Opin. Cell Biol.*, **7**, 697-706.

Ignelzi MA, Miller DR, Soriano P and Maness PF. (1994). *Neuron*, **12**, 873-884.

Kaplan KB, Bibbins KB, Swedlow JR, Arnaud M, Morgan DO and Varmus HE. (1994). *EMBO J.* **13**, 4745-4756.

Kaplan KB, Swedlow JR, Morgan DO and Varmus HE. (1995). *Genes Devel.*, **9**, 1505-1517.

Kaplan KB, Swedlow JR, Varmus HE and Morgan DO. (1992). *J. Cell Biol.*, **118**, 321-333.

Kellie S. (1988). *BioEssays*, **8**, 25-29.

Kellie S, Patel B, Wigglesworth NM, Critchley DR and Wyke JA. (1986). *Exp. Cell Res.*, **165**, 216-228.

Klewes L, Turley EA and Prehm P. (1993). *Biochem J.*, **290**, 791-795.

Kremer NE, D'Arcangelo G, Thomas SM, De Marco M, Brugge JS and Halegoua S. (1991). *J. Cell Biol.*, **115**, 809-819.

Kundra V, Soker S and Zetter BR. (1994). *Oncogene*, **9**, 1429-1435.

Lauffenburger DA and Horwitz AF. (1996). *Cell*, **84**, 359-369.

Laurent TC and Fraser JRE. (1992). *FASEB J.*, **6**, 2397-2404.

Lo SH and Chen LB. (1994). *Cancer Met. Rev.*, **13**, 9-24.

MacAuley A and Cooper JA. (1989). *Mol. Cell. Biol.*, **9**, 2648-2656.

Maness PF and Cox ME. (1992). *Semin. Cell Biol.*, **3**, 117-126.

Marchisio PC, Cirillo D, Zamborin-Zallone A and Tarone G. (1987). *Exp. Cell Res.*, **169**, 202-214.

Marrero MB, Schieffer B, Paxton WG, Schieffer E and Bernstein KE. (1995). *J. Biol. Chem.*, **270**, 15734-15738.

Matsumoto K, Matsumoto K, Nakamura T and Kramer RH. (1994). *J. Biol. Chem.*, **269**, 31807-31813.

Matsuyoshi N, Hamaguchi M, Taniguchi S, Nagafuchi A, Tsukita S and Takeichi M. (1992). *J. Cell Biol.*, **118**, 703-714.

McCarthy J and Turley EA. (1993). *Crit. Rev. Oral Biol. Med.*, **4**, 619-637.

Nakamura N, Tanaka J and Sobue K. (1993). *J. Cell Sci.*, **106**, 1057-1069.

Neet K and Hunter T. (1995). *Mol. Cell. Biol.*, **15**, 4908-1920.

Nermut MV, Eason P, Hirst EM and Kellie S. (1991). *Exp. Cell. Res.*, **193**, 382-397.

Nobes CD and Hall A. (1995). *Cell*, **81**, 53-62.

O'Connor TJ, Neufeld E, Bechberger J and Fujita DJ. (1992). *Cell Growth Differ.*, **3**, 435-442.

Ocheing J, Basolo F, Albini A, Melchiori A, Watanabe H, Elliot J, Raz A, Parodi S and Russo J. (1991). *Invasion Metas.*, **11**, 38-47.

Oddie KM, Litz JS, Balserak JC, Payne DM, Creutz CE and Parsons SJ. (1989). *J. Neurosci. Res.*, **24**, 38-48.

Okamura H and Resh MD. (1994). *Oncogene*, **9**, 2293-2303.

Okamura H and Resh MD. (1995). *J. Biol. Chem.*, **270**, 26613-26618.

Oude Weernink PA and Rijksen G. (1995). *J. Biol. Chem.*, **270**, 2264-2267.

Partin AW, Isaacs JT, Treiger B and Coffey DS. (1988). *Cancer Res.*, **48**, 6050-6053.

Ptasznik A, Traynor-Kaplan A and Bokoch GM. (1995). *J. Biol. Chem.*, **270**, 19969-19973.

Resh MD. (1990). *Oncogene*, **5**, 1437-1444.

Resh MD. (1994). *Cell*, **76**, 411-413.

Richardson A and Parsons JT. (1995). *BioEssays*, **17**, 229-236.

Rodgers W, Crise B and Rose JK. (1994). *Mol. Cell. Biol.*, **14**, 5384-5391.

Rodier J-M, Vallés AM, Denoyelle M, Thiery JP and Boyer B. (1995). *J. Cell Biol.*, **131**, 761-773.

Rudd CE, Janssen O, Prasas KVS, Raab M, da Silva A, Telfer JC and Yamamoto M. (1993). *Biochim. Biophys Acta*, **1155**, 239-266.

Sankar S, Mahoofi-Brooks N, Hu G and Madri JA. (1995). *Am. J. Pathol.*, **147**, 601-608.

Sargiacomo M, Sudol M, Tang Z and Lisant MP. (1993). *J. Cell. Biol.*, **122**, 789-807.

Sato K, Miki S, Tachibana H, Hayashi F, Akiyama T and Fukami Y. (1990).

Biochem. Biophys. Res. Commun., **171**, 1152-1159.

Savani RC and Turley EA. (1995). *Int. J. Tiss. Res.*, **17**, 141-151.

Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR and Parsons JT. (1994). *Mol. Cell. Biol.*, **14**, 1680-1688.

Sherman L, Sleeman J, Herrlich P and Ponta H. (1994). *Curr. Opin. Cell Biol.*, **6**, 726-733.

Soriano P, Montgomery C, Geske R and Bradley A. (1991) *Cell*, **64**, 693-702.

Stoker M and Gherardi E. (1991). *Biochim. Biophys. Acta*, **1072**, 81-102.

Stossel TP. (1993). *Science*, 260, 1086-1094.

Takeda H, Nagafuchi A, Yonemura S, Tsukita S, Behrens J, Birchmeier W and Tsukita S. (1995). *J. Cell Biol.*, **131**, 1839-1847.

Tarone G, Cirillo D, Giancotti FG, Comoglio PM and Marchisio PC. (1985). *Exp. Cell Res.*, **159**, 141-157.

Thomas JE, Aguzzi A, Soriano P, Wagner EF and Brugge JS. (1993). *Oncogene*, **8**, 2521-2529.

Thomas SM, P Soriano and Imamoto A. (1995). *Nature*, **376**, 267-271.

Toole BP, Goldberg RL, Chi-Rosso G, Underhill CB and Orkin R. (1989). *Biology of Hyaluronan. Ciba Found. Symp.*, **143**, 150-169.

Trahey M, Milley RJ, Cole GE, Innis M, Paterson H, Marshall CJ, Hall A and McCormick F. (1987). *Mol. Cell. Biol.*, **7**, 541-544.

Turley EA. (1989). *Biology of Hyaluronan. Ciba Found. Symp.*, **143**, 121-137.

Turley EA. (1992). *Cancer Metastasis Rev.*, **11**, 21-30.

Turley E and Auersperg N. (1989). *Exp. Cell Res.*, **182**, 340-348.

Turley EA, Austen L, Vandeligt K and Clary C. (1991). *J. Cell Biol.*, **112**, 1041-1047.

Turley EA, Brassel P and Moore D. (1990). *Exp. Cell Res.* **187**, 243-249.

Twamley-Stein GM, Pepperkok R, Ansorge W and Courtneidge SA. (1993). *Proc. Natl. Acad. Sci.*, **90**, 7696-7700.

Vallés AM, Tucker GC, Thiery JP and Boyer B. (1990). *Cell Regul.*, **1**, 975-988.

Warren SL and Nelson WJ. (1987). *Mol. Cell. Biol.*, **7**, 1326-1337.

Weng Z, Taylor JA, Turner CE, Brugge JS and Seidel-Dugan C. (1993). *J. Biol. Chem.*, **268**, 14956-14963.

Wu H and Parsons JT. (1993). *J. Cell Biol.*, **120**, 1417-1426.

Yang B, Entwistle, J, Zhang L and Turley EA. (1993). Manuscript in preparation.

Legends to Figures

Figure 1. ***Src and RHAMM are required for elevated cell motility in fibroblasts.*** **A.** The cell locomotion of 24 h, defined media cultures of wild-type, src (-/-) and src (-/-) fibroblasts transfected with c-src (c-src) in the presence of control (black bars), polyclonal (white bars) or monoclonal (hatched bars) anti-RHAMM blocking antibodies was analyzed over a 2 h period. Src (-/-) cells locomote at a significantly ($p < 0.001$) lower basal rate than the wild-type and c-src cells and are unaffected by the RHAMM blocking antibodies. Anti-RHAMM blocking antibodies significantly ($p < 0.001$) inhibit the cell locomotion of wild-type and c-src fibroblasts. **B.** Anti-RHAMM immunoblot analysis of total cell lysate from wild-type (lane **1**) and src (-/-) cells (lane **2**) illustrating that both cell lines express RHAMM (arrows). The anti-RHAMM polyclonal (R10.1) antibody recognized two RHAMM-specific bands that may represent glycosylation or splice variants (Entwistle *et al.*, 1995). M = molecular weight standards; 190, 125, 88, 65, 56, 38 and 33.5 kDa. **C.** Cell locomotion analysis of src (-/-) fibroblasts and those expressing src constructs reveals that src kinase is required for elevated cell locomotion. Src (-/-) fibroblasts expressing c-src or srcA locomote at a significantly higher rate ($p < 0.001$), but srcAM expression has no effect on cell motility and src251 expression only results in a small, but significant ($p < 0.05$), increase ($n = 69$- 80 cells per group).

Figure 1A.

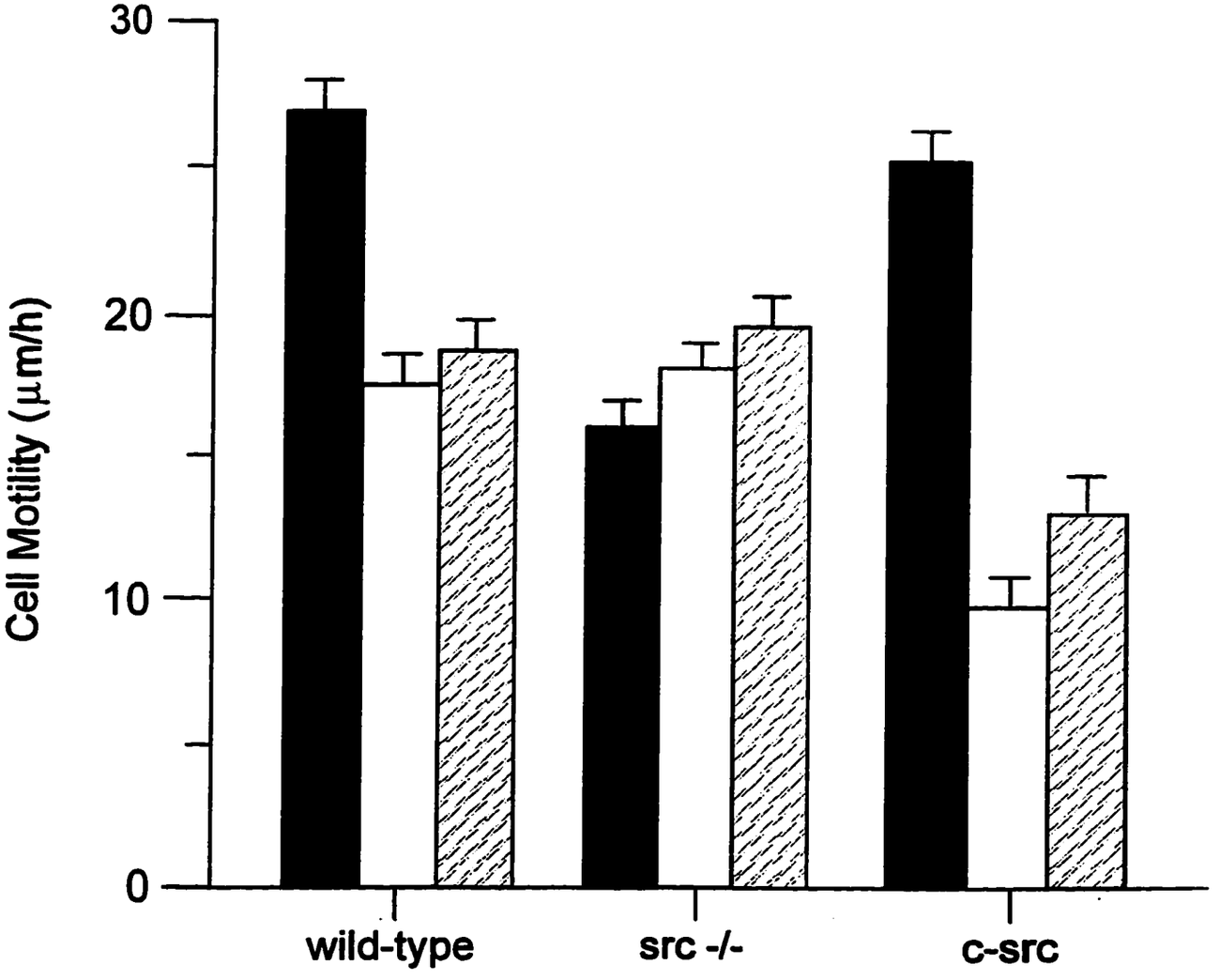


Figure 1. B.

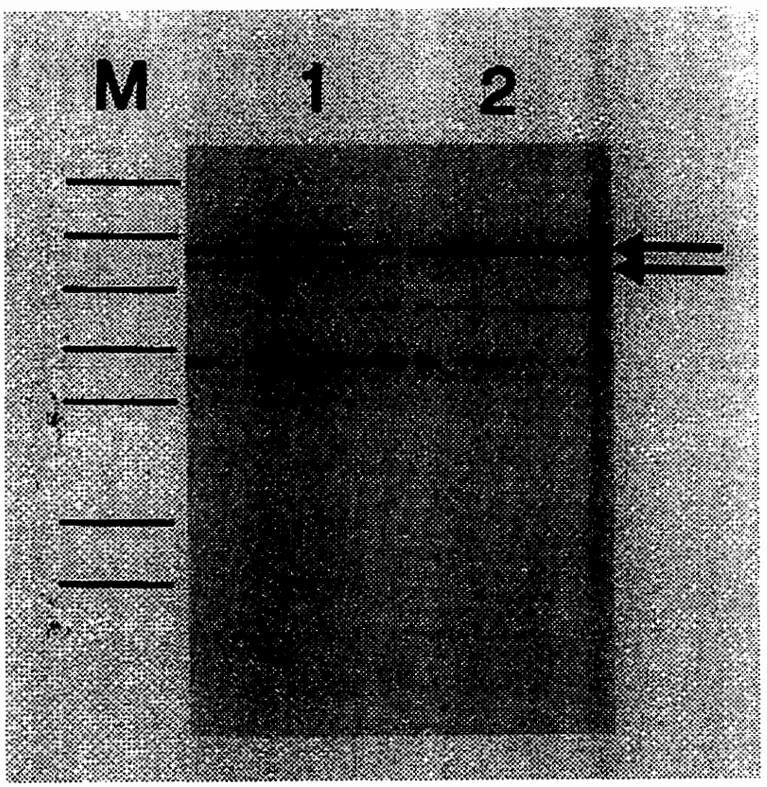


Figure 1C.

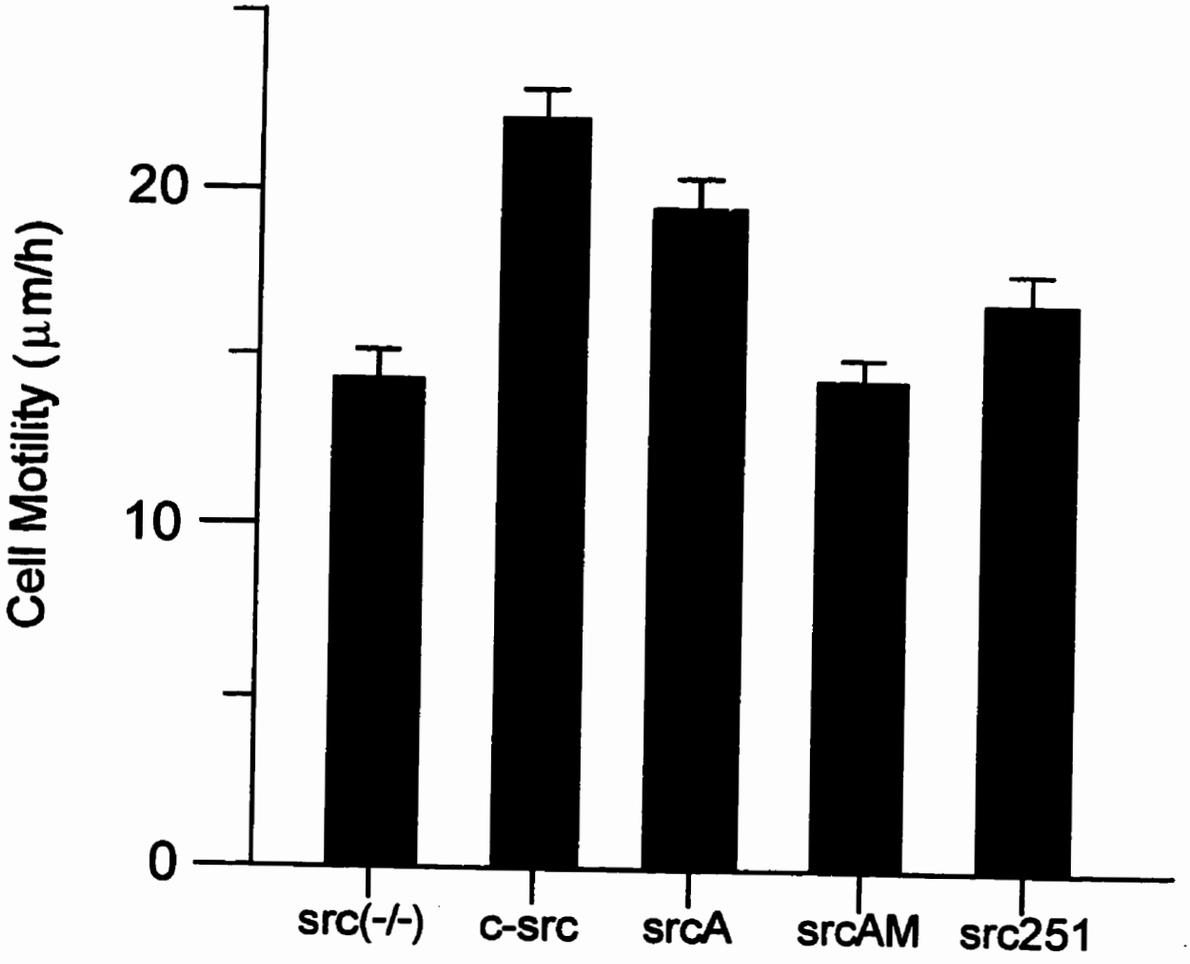


Figure 2. ***Dominant negative src inhibits cell motility in RHAMM1v4-transformed fibroblasts.*** **A.** Random cell locomotion analysis of *RHAMM1v4*-10T½ fibroblasts and the same cells expressing srcAM in defined media reveals that expression of srcAM significantly ($p < 0.001$) inhibits cell locomotion (n=100-116 cells per group). **B.** Immunofluorescent staining of these fibroblasts with anti-vinculin and phalloidin reveals no obvious difference between the focal adhesions and actin fibers of *RHAMM1v4*-10T½ cells and those expressing dominant negative srcAM. 1 inch = 25 μm.

Figure 2A.

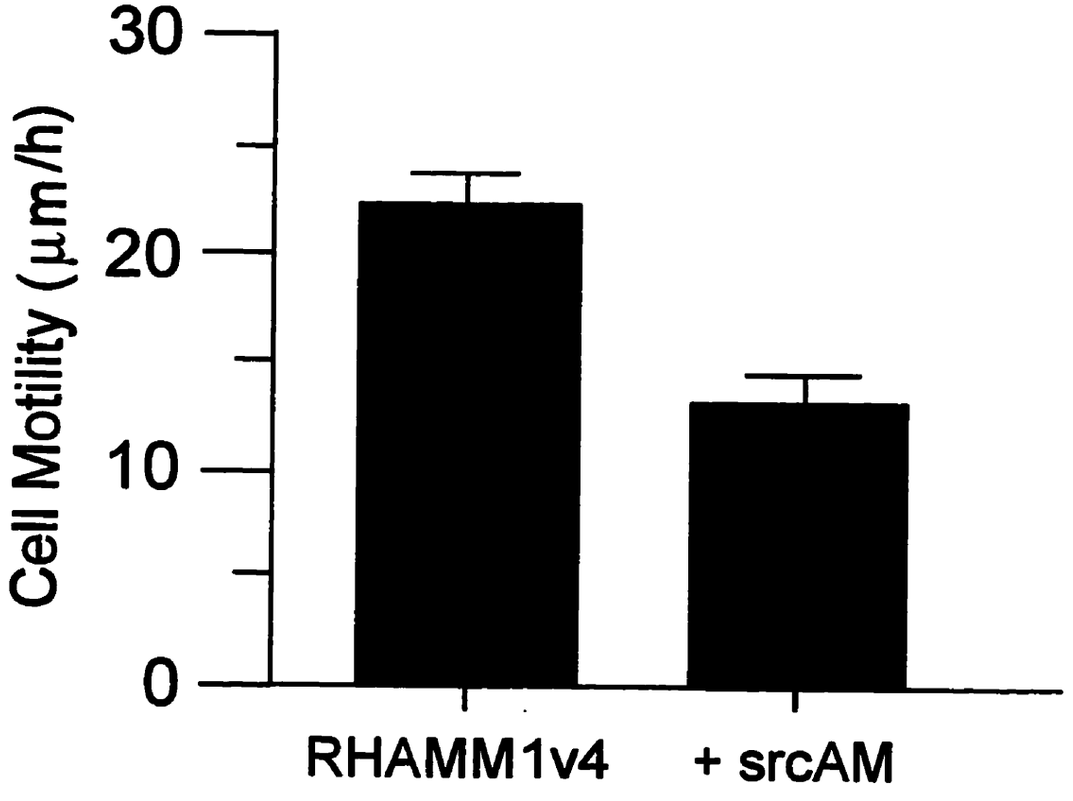


Figure 2B

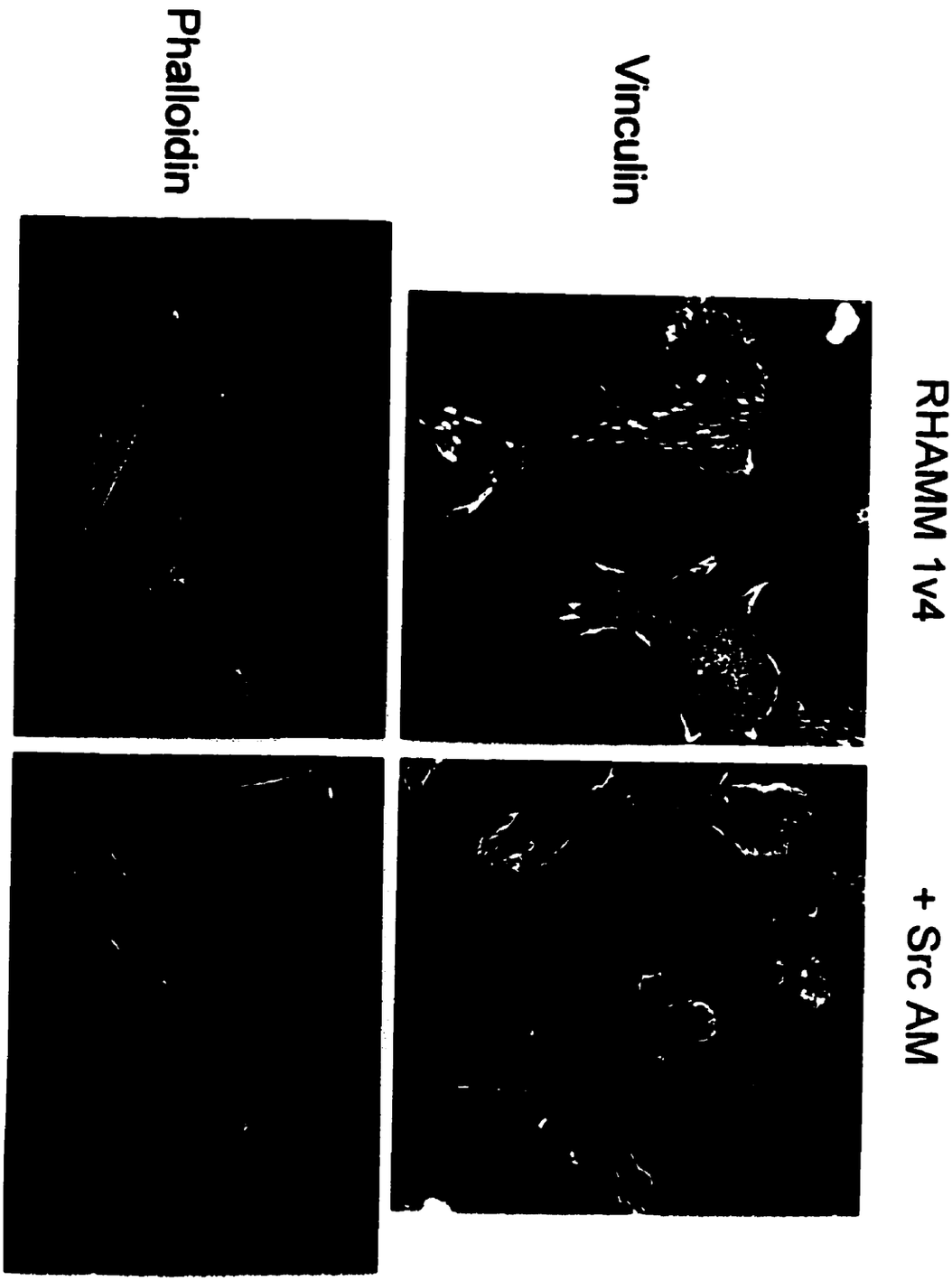


Figure 3. ***Src peptide inhibitor and src substrate peptide inhibit HA induced cell motility.*** The cell motility of HA (10 ng/ml) or control treated (black bars) *ras*-transformed fibroblasts in defined media was examined after treatment with peptides covalently linked to the Penetratin 1 internalization vector. HA addition results in a significant ($p < 0.01$) increase in cell motility while a src peptide inhibitor (peptide 1) and a src substrate peptide (peptide 2) prevented HA induced cell motility. A typical trial is shown ($n = 30$ cells per group).

Figure 3.

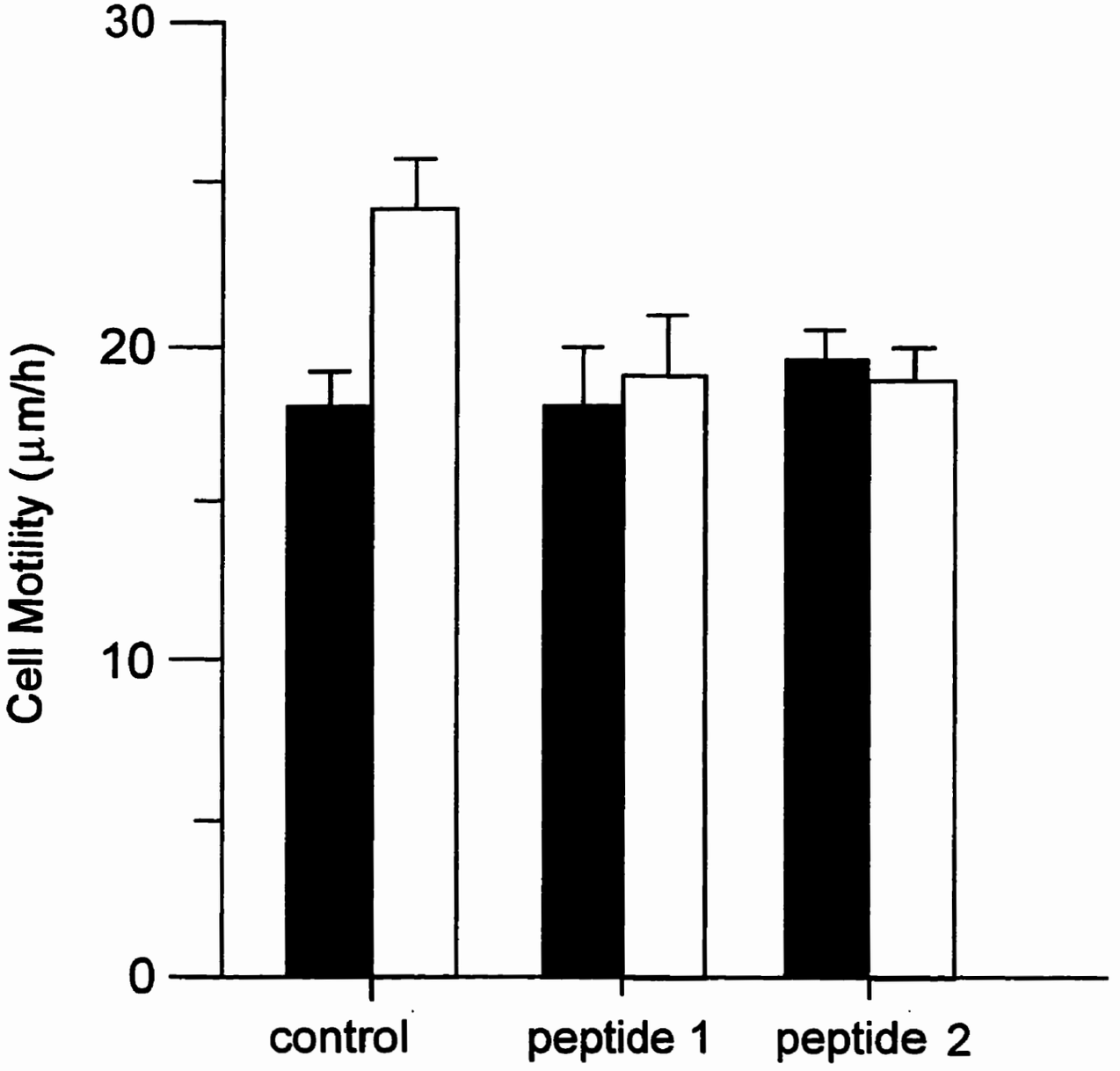


Figure 4. ***Motility in v-src transformed fibroblasts does not require RHAMM.*** **A.** Cultures of 10T½ and v-*src*-10T½ fibroblasts in defined media were treated with a monoclonal anti-RHAMM blocking antibody (white bars) or a non-blocking isotype control antibody (black bars) and subjected to random cell motility analysis. Transformation with v-src resulted in a significant ($p < 0.001$) increase in cell motility over the parental cell line. RHAMM blocking antibody significantly ($p < 0.001$) decreased the cell motility of the parental fibroblasts, but had no effect on v-*src*-transformed cell locomotion (n=73-120 cells for each group). **B.** 10T½ fibroblasts and 10T½ fibroblasts that express little RHAMM (U21, 10% of parent line) were transformed with v-src. Focal contacts were detected by anti-vinculin antibodies and actin filaments were detected with RITC-phalloidin. v-*Src* caused disassembly of actin stress fibres and focal constructs in the parent 10T½ line expressing RHAMM but not in cells expressing little RHAMM.

Figure 4. A.

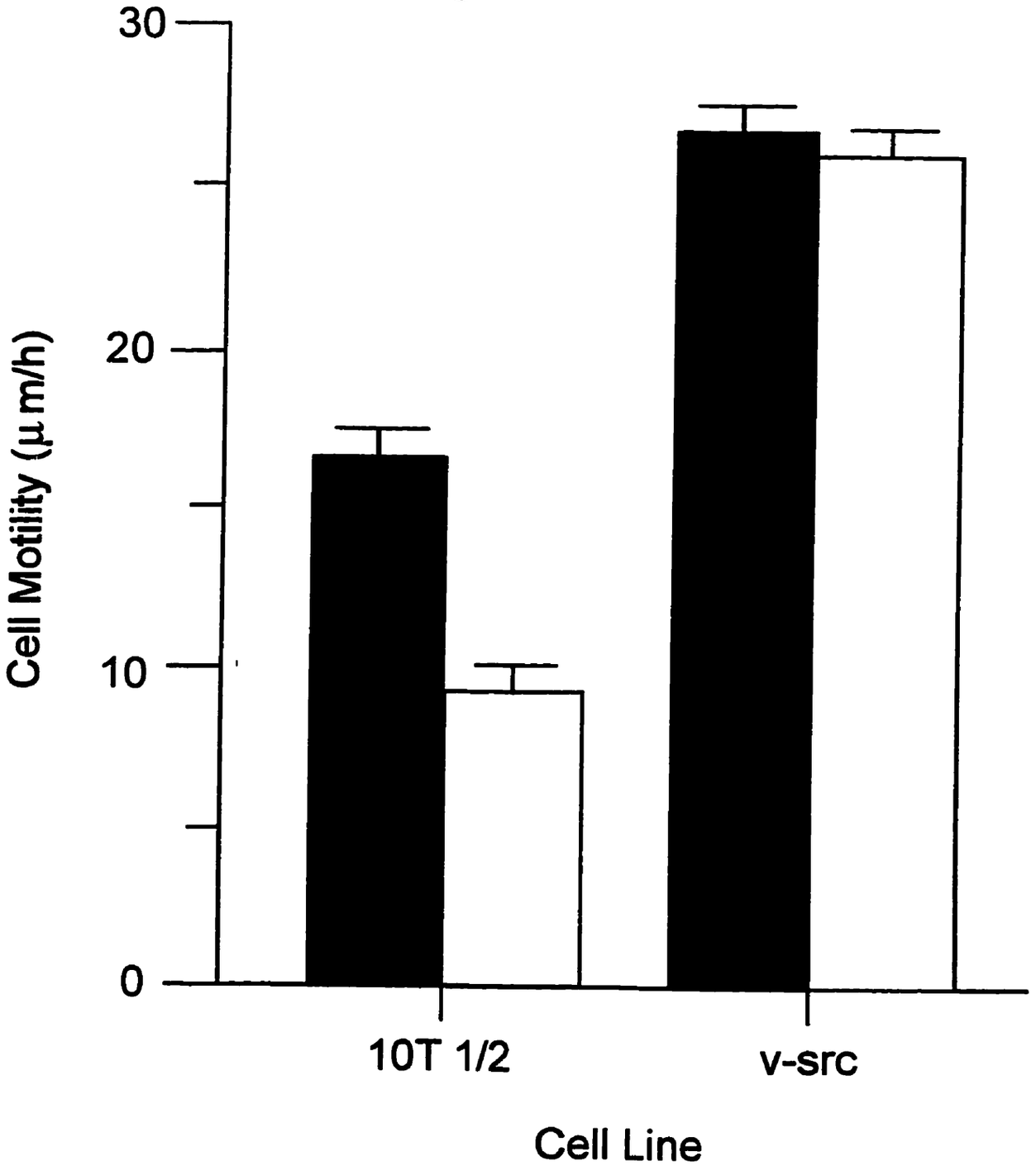


Figure 4B

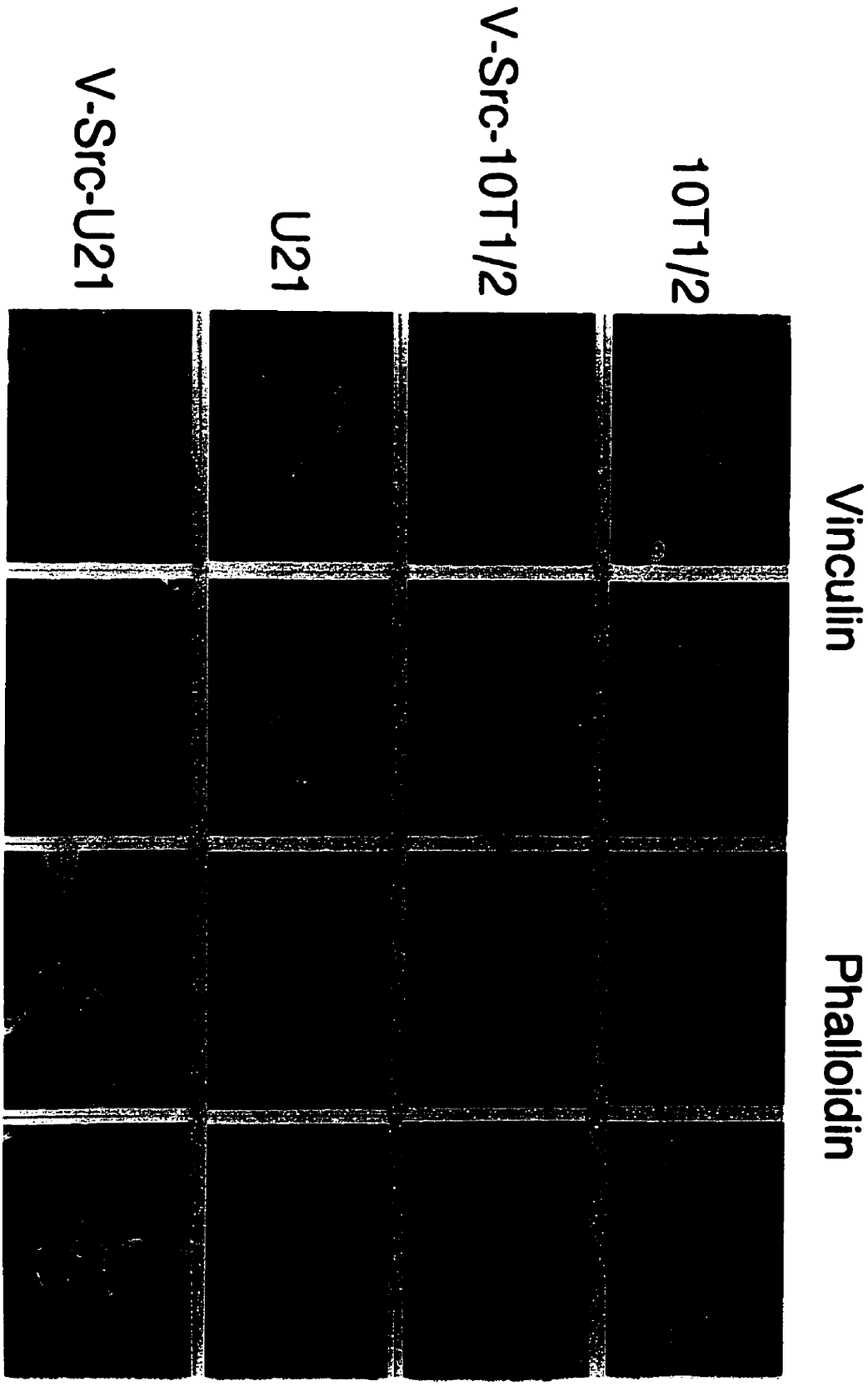


Figure 5. ***RHAMM and src co-localize and co-associate.*** **A.** *Ras*-transformed fibroblasts were fixed at a time when the cells are highly motile (24 h after subculture, in growth medium) and stained by indirect immunofluoresce with both rabbit polyclonal anti-RHAMM (**a-d**) and mouse monoclonal anti-src 2-17 (**f-i**) followed by anti-rabbit-TRITC and anti-mouse-FITC. The double staining indicates a variable co-localization of RHAMM and src, several of which are displayed. Rabbit (panel **e**) and mouse antibody (panel **f**) controls are shown. 1 inch = 25 μm. **B.** Src immunoprecipitates (anti-src 2-17; lanes **3** and **7**) from CHAPS extracts of *ras*-transformed fibroblasts were subjected to immunoblot analysis with a polyconal anti-peptide RHAMM antibody (lanes **1-4**) or with antibody pre-incubated with peptide (lanes **5-8**). Controls were as follows: whole cell extract (lanes **1** and **5**), control IgG immunoprecipitate (lanes **2** and **6**) and mock immunoprecipitates (extraction buffer and anti-src; lanes **4** and **8**). Approximately 2% of the RHAMM protein in the cell extracts co-precipitated with anti-src. Similar results were obtained with MAb 327 anti-src antibody (data not shown). Molecular weight markers (M) were 190, 125, 88, 65, 56, 38 and 33.5 kDa.

Figure 5A

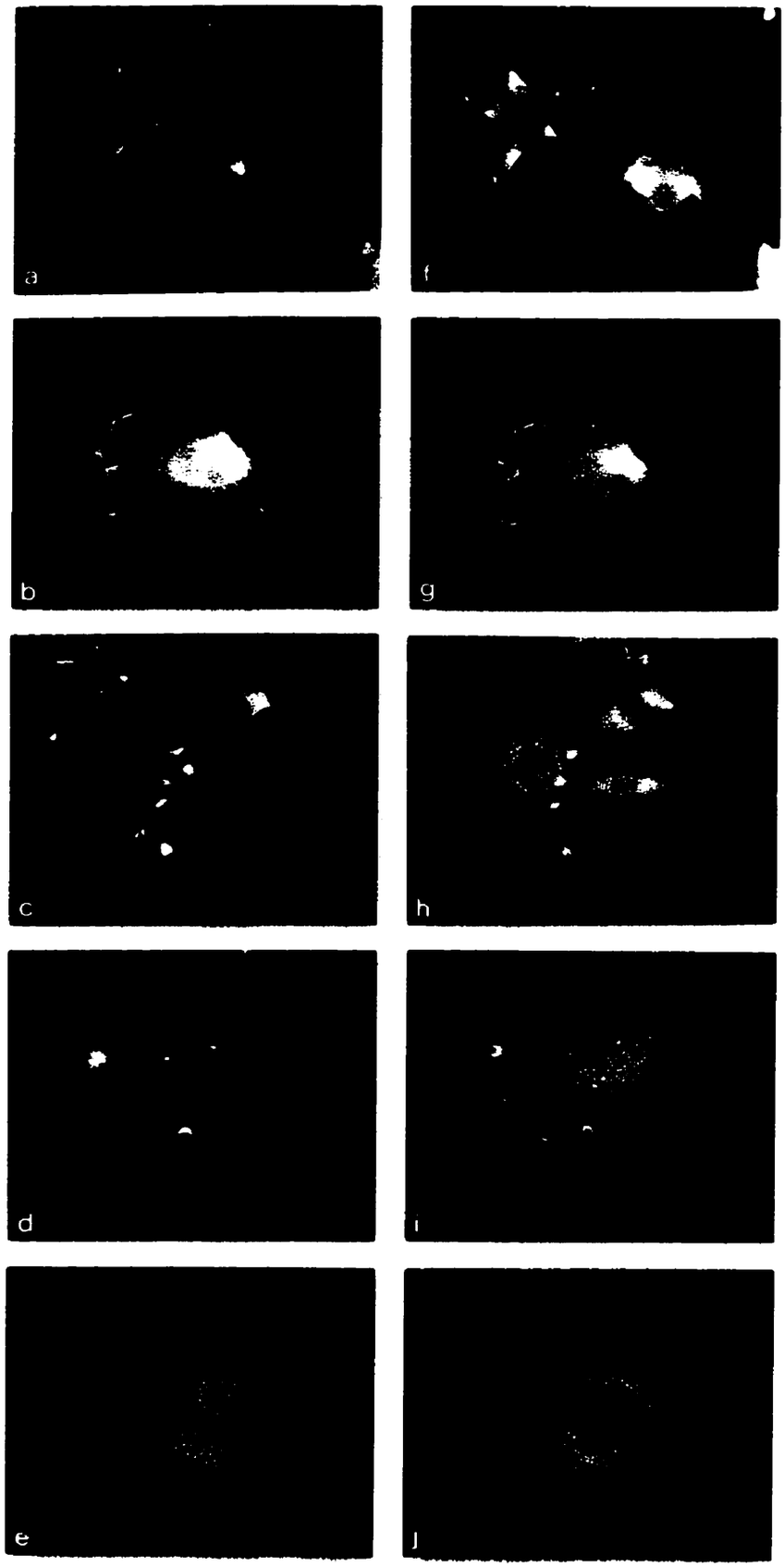


Figure 5. B.

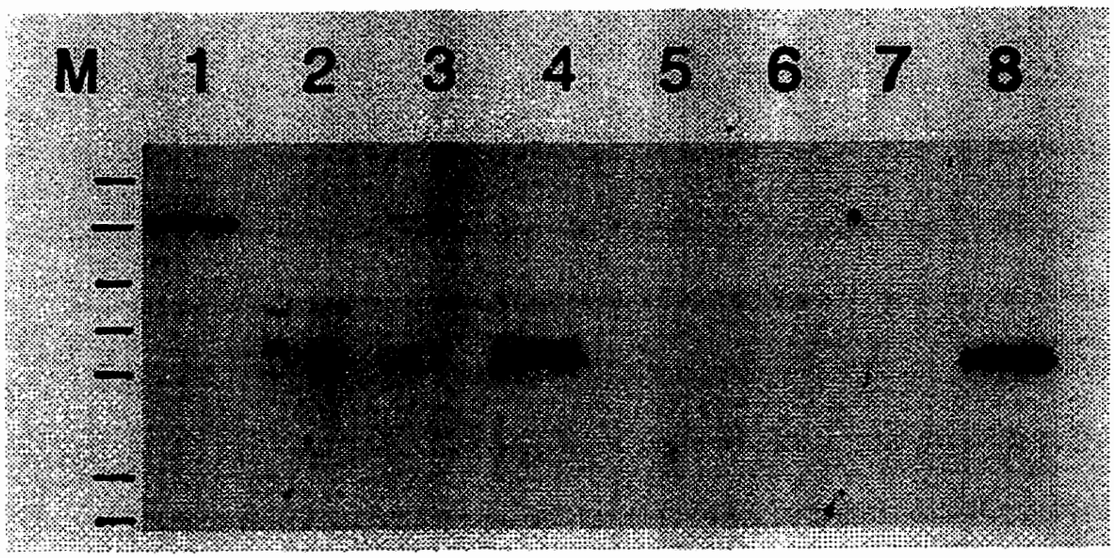


Figure 6. ***Src kinase activity corresponds to RHAMM induced cell motility.*** **A.**. The random cell locomotion rates of 24 h cultures of fibroblasts were determined using the Image-1 computer time-lapse analysis system. The *ras*- and *RHAMM1v4*-transformed have elevated RHAMM expression and elevated cell motility rates compared to parental 10T½ cells (for each cell line n=90 cells in growth media; black bars, left axis). Immunoprecipitation of equal amount of src kinase from these cell lines followed by a src peptide kinase assay (white bars, right axis) shows that src activity correlates with cell motility (n=3 assays). **B.** Treatment of *ras*-transformed fibroblasts in defined media with stimulatory anti-RHAMM antibody (Hall *et al.*, 1994) results in a transient increase in src kinase activity. Src immunoprecipitates from RIPA buffer lysates of fibroblasts stimulated with anti-RHAMM for 20 sec, 1 min, 5 min or with preimmune serum for 1 min (Control) were subjected to a src autokinase-gel assay. Src autophosphorylation and phosphorylation of the immunoglobulin present were measured using a Phosphoimager and the amount of phosphorylation presented in arbitrary units (n=3 assays). A typical autoradiograph is shown in the inset. Equal loading was assessed by determining the amount of src immunoprecipitated using a Western blot assay (data not shown).

Figure 6A.

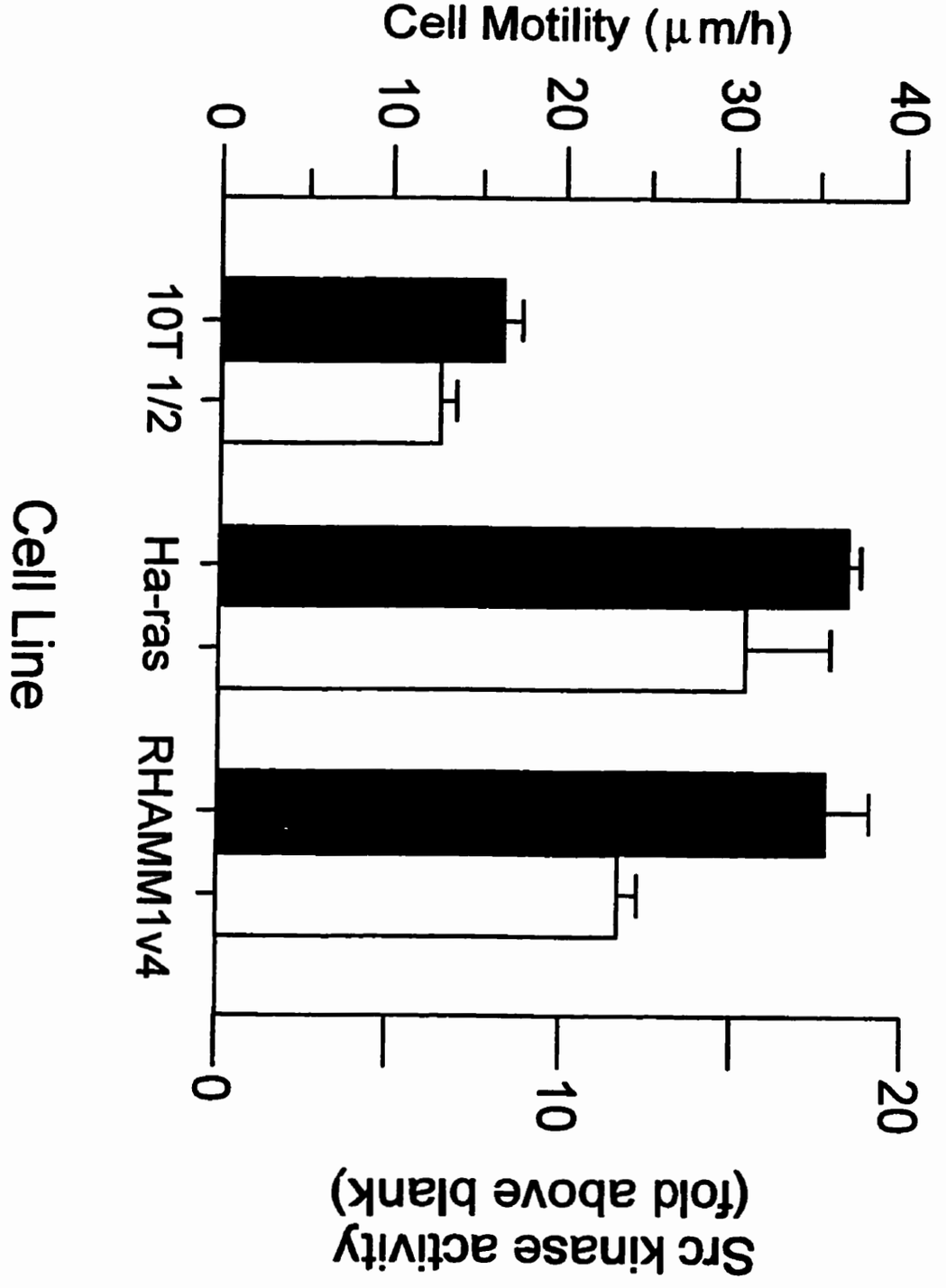

Cell Motility (μm/h)
0
10
20
30
40
10T 1/2
Ha-ras
RHAMM1v4
Cell Line
Src kinase activity
(fold above blank)
0
10
20

Figure 6B.

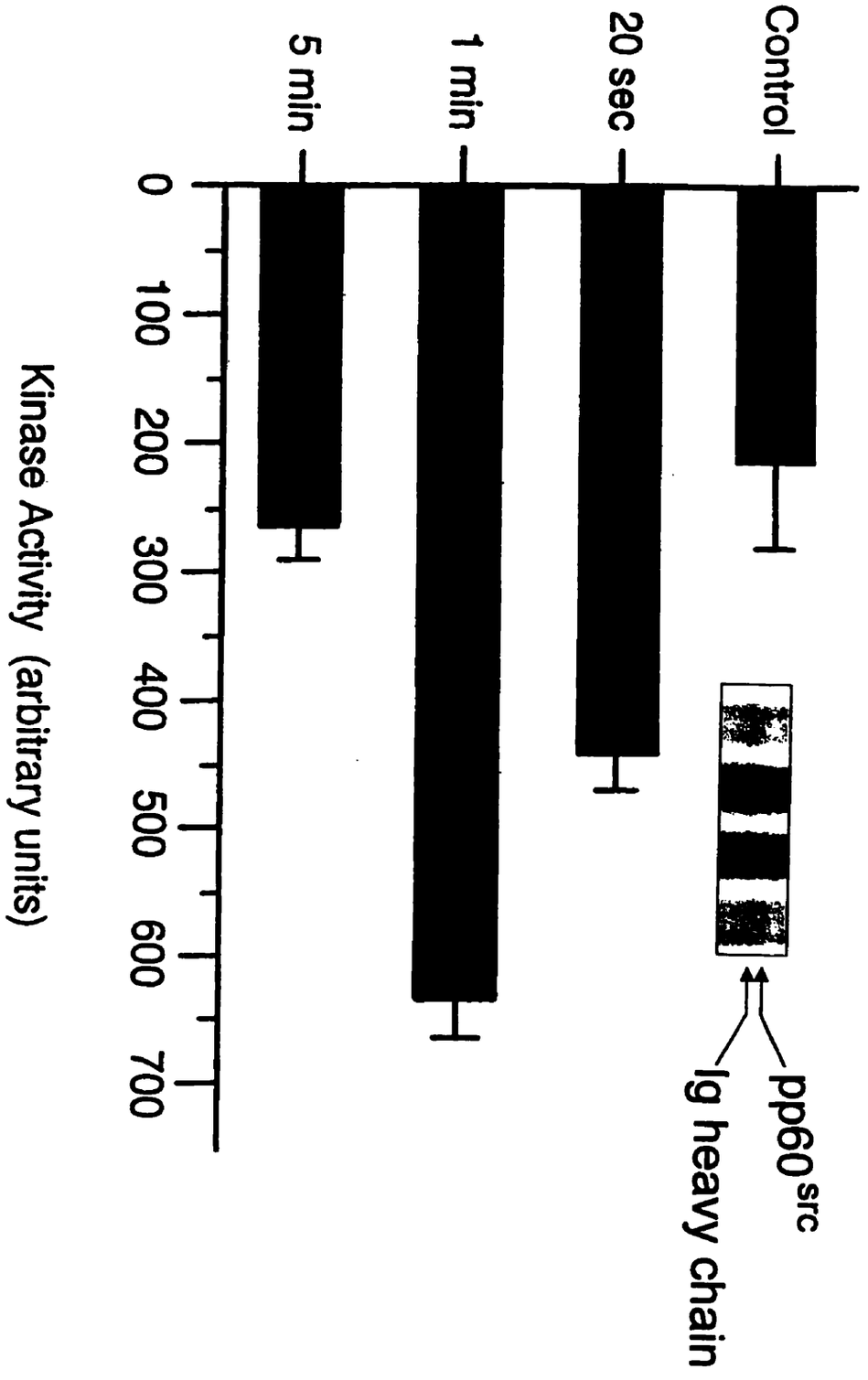
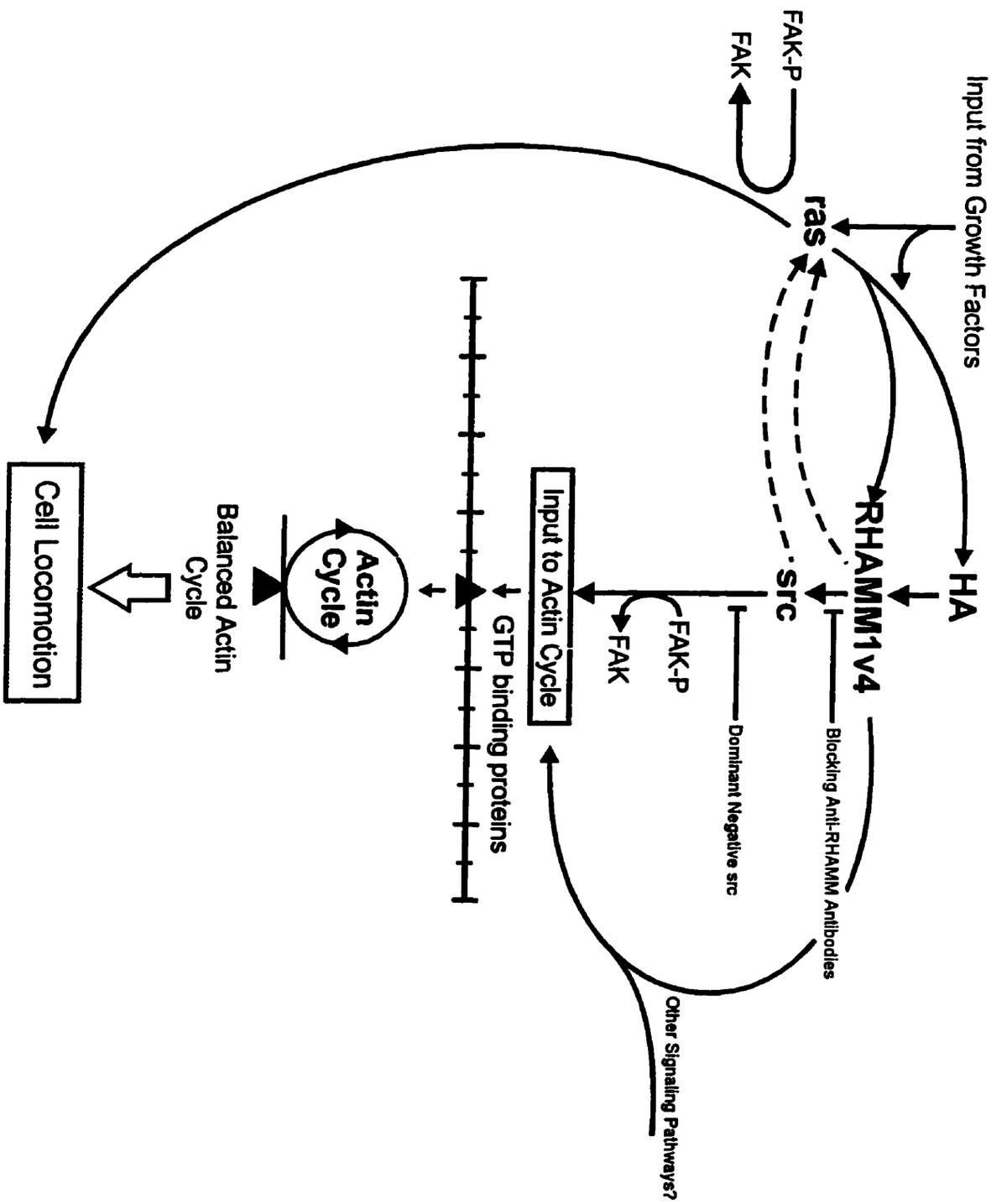

Control
20 sec
1 min
5 min
0
100
200
300
400
500
600
700
Kinase Activity (arbitrary units)
pp60 src
Ig heavy chain

Figure 7. ***Model: HA/RHAMM/src signaling promotes cell motility via input into the actin cycle.*** The model presented here represents one signaling pathway that we have shown to regulate cell locomotion. Activation of src via HA:RHAMM may signal alterations in actin filament and cell-matrix adhesion structure to allow for the cycles of actin polymerization/depolymerization required for cell locomotion. Several different signaling cascades may contribute to the regulation and balance of the actin cycle to influence cell motility and adhesion (see text). Other pathways stemming from ras regulate gene expression and proliferation, and may regulate cell locomotion as well.

Figure 7


Input from Growth Factors
FAK-P
FAK
ras
HA
RHAMM1v4
src
Blocking Anti-RHAMM Antibodies
Dominant Negative src
FAK-P
FAK
Other Signaling Pathways?
Input to Actin Cycle
GTP binding proteins
Actin Cycle
Balanced Actin Cycle
Cell Locomotion

Summary

Summary

This thesis has addressed the role of the hyaluronan receptor RHAMM in tumor cell locomotion, tumorigenesis and metastasis. In the previous chapters RHAMM has been shown to be an important regulator of cell motility, adhesion, cytoskeletal organization, cell growth and cellular transformation. As a brief overview, it has been shown that: RHAMM is capable of signaling cell locomotion via tyrosine phosphorylation pathways that affect cytoskeletal assembly and cell adhesion; that RHAMM is instrumental to *ras*-induced cell motility and *ras*-transformation; that when overexpressed, RHAMM can act as an oncogene and confer metastatic ability; that the HA binding function of RHAMM is a requirement for its activities; and that RHAMM signaling is linked to the proto-oncogene src, where src is required for RHAMM induced cell motility. In the introduction, the previous connections between HA and transformation and metastasis were outlined. Here, it is demonstrated that HA may elicit its effects via signaling through its receptor RHAMM.

Transformation from a normal cell phenotype to a tumorigenic and metastatic one, involves changes in cell shape, changes in the adherence of cells to the ECM and to surrounding cells, changes in the ability to migrate and invade and changes leading to deregulated growth. Adhesive interactions at focal adhesions can play an important role in all of these events. Indeed, a distinct relationship between adhesion, cell migration and growth has emerged. Notably, cells that are strongly adherent to the ECM, with large stable focal adhesions, are restricted in their growth

and motility. Whereas, cells that are moderately adhesive to the ECM, with smaller more dynamic adhesions, have higher growth and motility rates, in general. Transformed cells fall into this second category. They are able to rapidly form new adhesions and dissolve old adhesions, allowing for elevated locomotion and growth. The overexpression of RHAMM results in such a change in adhesion phenotype. The change from highly adhesive, less motile cells to moderately adhesive, more motile cells may result from HA:RHAMM induced signaling within focal adhesions.

One model of how HA:RHAMM signaling could affect cell shape and adhesion, and hence, cell locomotion and transformation, is presented in the figure. This figure is presented as an over simplification, where cell shape and adhesion changes are generated by input into the actin cycle. The actin cycle refers to the cytoskeletal solation and gelation, and actin filament cross-linking, capping, severing and attachment to the membrane that are essential events for cell locomotion. The continuous gelation and reassembly of actin filaments and associated protein complexes are fundamental requirements for membrane protrusion, for the formation of new adhesions at the front of the cell and for the retraction/release of adhesions at the trailing end. The precise balance of the actin cycle by a number of signaling cascades is intimately connected to, if not instructive to, cell locomotion. The actin cycle also contributes to cell growth. As mentioned above, the ideal state for cell growth is a moderate adhesive state, with a "balanced" actin cycle. In the model presented here, HA:RHAMM signaling balances the actin cycle and allows the assembly and disassembly required for cell locomotion and cell growth.

The studies presented in this thesis place RHAMM as a central player in tumor cell locomotion (see Figure). In *ras*-transformed cells, mutant *ras* expression results in an elevated level of the RHAMM1v4 isoform and elevated HA production. Evidence presented in this thesis suggest that HA:RHAMM signaling pathways then lead to changes in cell motility and cell growth. For instance, it has been shown that HA:RHAMM signaling can promote src activity and a transient phosphorylation of FAK. Src and FAK, individually or as a complex, could influence actin fiber formation/gelation, membrane anchoring/release and the initial formation of cell-matrix adhesions, i.e. input into the actin cycle, by phosphorylating cytoskeletal protein targets (see Figure). Addressing what these targets are and the role they play in cell locomotion is an important question. Likely targets include cortactin which can promote the anchoring of actin filaments into the plasma membrane, and tensin which can affect the aggregation of actin filaments at focal adhesions. Thus, in *ras*-transformed cells, HA:RHAMM signaling via src and FAK can affect the cytoskeleton, and cell locomotion.

In addition to src kinase, other signals from RHAMM or *ras* could contribute to focal adhesion turnover. For example, the convergence of src and *ras* pathways at p190RhoGAP may regulate actin assembly/disassembly by influencing rho. Rho, rac and cdc42 are a family of small GTPases like ras that act as molecular switches regulating lamellar extension and adhesion. The constant cycling of focal adhesion turnover, phosphorylation/dephosphorylation and the actin assembly/disassembly suggest that these molecular switches are involved in cell locomotion. The pathway

shown in this model places RHAMM in an ideal position to influence these molecular switches and have further input into the actin cycle. Reagents and mutants that block rho, rac and cdc42 activity are available to address their role in HA:RHAMM signaling.

In this model, those events contributing to HA:RHAMM induced elevated cell motility also contribute to tumorigenesis. Both during transformation and in preparation of elevated cell locomotion, there is a change from larger focal adhesions to podosomes, changes that require tyrosine phosphorylation and may involve src and FAK. RHAMM induced events at focal adhesions may also result in signaling to the nucleus, signals that affect gene expression and cell proliferation. Indeed, there is now evidence that RHAMM influences the MAP kinase pathway and that intracellular HA and RHAMM may stimulate nuclear events. What remains to be determined is the whether focal adhesion turnover is necessary for HA:RHAMM induced transformation and tumorigenesis. Using various RHAMM, ras, and src mutants, it may be possible to determine which signals are important for cell motility and which are important for cell growth. By affecting focal adhesion formation with different reagents, the involvement of focal adhesion signaling in RHAMM induced tumorigenesis may be determined.

Finally, the above model identifies just some of the possibilities for future work on HA:RHAMM cell motility and *RHAMM*-transformation. In addition to identifying the protein targets of src and FAK in the HA:RHAMM motility pathway, determining the involvement of the small GTPases, rho, rac and cdc42 and

addressing whether focal adhesion turnover is required for transformation and tumorigenesis, a number of other questions remain to be answered. For example, the physical link between RHAMM and src is weak at best. Further work is required to identify how RHAMM and src associate and to identify whether other proteins are involved. In addition, RHAMM has been found to localize to focal adhesions. It is important to identify which isoform of RHAMM localizes to adhesion and to determine if this isoform is responsible for signaling cell motility, or whether other isoforms are also involved. As well, RHAMM is not the only HA receptor, many cell also express CD44. It will be interesting to identify any interplay between RHAMM and CD44. Finally, this thesis has looked at RHAMM in a mouse fibroblast system. Work in human cells and animal models will determine the role for RHAMM in tumorigenesis and other physiological and pathological systems in vivo. Many of these questions are currently being addressed, and will be the topic of future theses.

Figure: Model: Role of RHAMM in transformation and tumor cell locomotion. This model depicts how HA/RHAMM/Src signaling can contribute to cell locomotion and cell growth by promoting changes in the actin cycle. The precise balance of the actin cycle by a number of signaling cascades is intimately connected, if not instructive, to both cell locomotion and cell growth. Without actin cycling, or in the presence of net cytoskeletal assembly, the cell is arrested in terms of movement and can not undergo cell proliferation. When the cytoskeleton is actively cycling from gelatinous to soluble (gel-sol transition), changes in cell shape allow cell movement, for migration and cell division. Furthermore, the gel-sol state allows signaling proteins to meet and separate and allows the movement of proteins from compartment to compartment, cytosol to nucleus. When net cytoskeletal disassembly occurs, the cell rounds up and lifts from the surface. Normally in such a "sol" state, cell division cannot occur, and neither can locomotion without substrate attachment or mechanism to create tension. In this model, HA:RHAMM signaling, together with input from several other pathways, affects the balance of the actin cycle and contributes to cell locomotion and growth. Here, HA interacts with RHAMM to promote src activity and src, in turn may influence downstream events that affect the actin cycle. Furthermore, growth factors that signal via ras can influence this pathway by determining the availability of enzyme substrates, or by altering HA production or RHAMM expression. All of these pathways combine and contribute to the actin state of assembly/disassembly, and hence to locomotion and growth.

Summary Figure