Ral-GTPases as Calmodulin Binding Proteins in Signal Transduction Pathways

By

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

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ABSTRACT

Ral GTPases may be involved in calcium (Ca^{2+})/calmodulin (CaM)-mediated intracellular signalling pathways because RalA and RalB are activated by Ca^{2+}, and RalA binds CaM *in vitro*. Examining Ral/CaM interactions, we demonstrate by yeast two hybrid (Y2H) analyses that both Rals interact directly but differentially with CaM *in vivo*. Co-immunoprecipitation experiments determined CaM and RalB form complexes in human platelets. *In vitro* pull-down experiments in human platelets showed endogenous Ral or CaM interact with recombinant CaM- or Ral-fusion proteins, respectively, in a Ca^{2+}-dependent manner. Truncated Ral constructs determined *in vitro* and *in vivo* that RalA has an additional CaM binding domain to that previously described, that although RalB binds CaM, its C-terminal region is involved in partially inhibiting this interaction, and that *in vitro* RalA and RalB have an N-terminal Ca^{2+}-independent and a C-terminal Ca^{2+}-dependent CaM binding domain. Functionally, *in vitro* Ral-GTP pull-down experiments determined that CaM is required for the thrombin-induced activation of Ral in human platelets. We propose that differential binding of CaM by RalA and RalB may underlie possible functional differences between the two GTPases, and that CaM is involved in the regulation of the activation of Ral-GTPases. Ral-GTPases are involved in the induction of DNA synthesis and the control of cell proliferation, and are essential for complete Ras-mediated oncogenic growth and morphologic transformation in human cells. These events are mediated by Ras through RalGEFs. However, Ral is also activated via Ras-independent RalGEFs and other pathways. Ral-GTPases are activated by epidermal growth factor (EGF) and are required for EGF-induced mitogenesis. We determined that Ral activation by EGF in A7r5 cells is dependent on Ca^{2+}, CaM, PLC,

PKCδ and the Ras-dependent RalGEFs, but not the Ras-independent RalGEF, BCAR3/AND-34. In HeLa cells, EGF-induced Ral activation requires Ca^{2+} and BCAR3/AND-34, while being only partially dependent on CaM, and not requiring Ras-dependent RalGEFs or PKC. In HeLa and MCF7 cells, the EGF-induced activation of RalB but not RalA requires PLC. Ral activation is inhibited in HeLa and unaffected in A7r5 cells by BCAR1/$p130^{Cas}$, an inhibitor of BCAR3/AND-34. In MCF7 cells, BCAR1/$p130^{Cas}$ enhances basal and EGF-induced Ral activation, which also requires CaM. Our results suggest that regulation of, and the RalGEFs activated in, EGF-induced Ral activation varies with cell type and environment. These findings may have implications in cell growth, proliferation and tumorigenesis. To determine additional functions for Ral, we searched for novel Ral binding proteins by screening a human testis cDNA expression library with recombinant RalB. We discovered the β1 subunit of Na^+/K^+-ATPase (β1) interacts directly with RalA and RalB *in vitro* and *in vivo* via Y2H and *in vitro* binding and pull-down assays. Na^+/K^+-ATPase functions as both an ion pump and a signal transducer. Ouabain and other cardiac glycosides partially inhibit Na^+/K^+-ATPase which activates hypertrophic growth pathways via c-Src/EGF receptor and intracellular signalling pathways. These include Ras/MEK/ERK and Ral/RalGDS cascades. Using Ral-GTP pull-down assays in A7r5 and HeLa cells, we show ouabain minimally activates Ral but inhibits the EGF-induced activation of Ral in A7r5 but not HeLa cells, and that long-term ouabain treatment downregulates Ral, β1, CaM and Ras protein expression. CaM is required for the ouabain-induced activation of c-Src, an effect which is inhibited by Ral. We propose Ral is involved in the signal transduction pathways

of Na^+/K^+-ATPase and we provide a molecular mechanism connecting Ral to cardiac hypertrophy.

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List of Abbreviations

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
AD	activation domain
AEBSF	4-[(2-aminoethyl)]-benzene sulfonyl fluoride
AGE	agarose gel electrophoresis
Amp	ampicillin
ANF	atrial natriuretic factor
AP-1	activator protein-1
AP2	adaptor protein complex-2
BAPTA-AM	1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate
BCAR1/3	breast cancer anti-estrogen receptor locus 1 and 3
BD	binding domain
Ca^{2+}	calcium
CaM	calmodulin
CaM BD	CaM-binding domain
CaMBP	CaM binding protein
Cas	Crk-associated substrate, as in $p130^{Cas}$
CNBr	cyanogen bromide
Crk	v-Src sarcoma virus CT10 oncogene homolog (avian)
CSM	canine sarcolemmal membrane preparation
c-Src	oncogene, homolog of Rous sarcoma virus (avian)
CT-1	Cardiotrophin-1
DAG	1,2-diacylglycerol

DMEM	Dulbecco's modified eagle's medium
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EH	epsin homology
ER	endoplasmic reticulum
ERK	extracellular-regulated kinase
FN	fibronectin
GAP	GTPase activating protein
GPCR	G-protein-coupled receptor
GR	glucocorticoid receptor
GSH	glutathione
GST	glutathione *S*-transferase
HA	haemagglutin
HCl	hydrochloric acid
IGF-1	insulin-like growth factor 1
IP_3	inositol-1,4,5-triphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
JNK	Jun amino-terminal kinase
K^+	potassium
KCl	potassium chloride
LB	Luria-Bertani
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site

MEK	MAPK kinase
MEM	minimum essential medium with Earle's salts
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MMP	matrix metalloproteases
MR	mineralocorticoid receptor
Na^+	sodium
NaH_2PO_4	sodium dihydrogen phosphate
$NaHCO_3$	sodium bicarbonate
NGF	nerve growth factor
PAR1/4	protease-activated receptors 1 and 4
PBS	phosphate buffered saline
PDA	phorbol 12, 13-diacetate
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonylfluoride

POB1	partner of RalBP1
PP2	a specific Src kinase inhibitor
PTK	protein tyrosine kinase
PVDF	polyvinylidene difluoride
RalBP1	Ral binding protein 1
RalGDS	Ral GDP dissociation stimulator
RalGEF	Ral guanine nucleotide exchange factor
RalGPS	(RalGEFs with pleckstrin homology [PH] domain and Src homology 3 [SH3] binding motif)
Ras	regulator of G-protein signalling
REPS1	RalBP1-associated Eps-homology domain protein
RIP1	Ral interacting protein 1
RIPA	radioimmune precipitation buffer
RLIP76	76 kDa Ral interacting protein
ROS	reactive oxygen species
RRBD	Ral binding domain of RIP1
SD	minimal synthetic dropout
SD/-HALT	SD lacking histidine, adenine, leucine and tryptophan
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SH3	Src homology 3
Shc	Src homology-containing
skACT	skeletal alpha actin
SOS	son of sevenless

STAT3	signal transducer and activator of transcription-3
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
Y2H	Yeast Two-Hybrid
$\alpha 3$	$\alpha 3$ subunit of Na^+/K^+-ATPase
$\beta 1$	$\beta 1$ subunit of Na^+/K^+-ATPase

1. INTRODUCTION

The Ral proteins, RalA and RalB, are small GTPases that belong to the Ras superfamily of low molecular mass GTP-binding proteins (Chardin and Tavitian, 1986, 1989). They are involved in an increasing number of signal transduction pathways and cell proliferation (Feig *et al.*, 1996; Reuther and Der, 2000), and are essential in human cells (Hamad *et al.*, 2002; Boettner and Van Aelst, 2002) for Ras-induced oncogenic growth and morphologic transformation (Urano *et al.*, 1996; White *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997; Webb *et al.*, 1998) and induction of DNA synthesis (Miller *et al.*, 1997). The Ral pathway is involved in Ras-induced anchorage-independent growth (Yamazaki *et al.*, 2001) and in cell invasion and metastasis (Yamazaki *et al.*, 2001; Okan *et al.*, 2001). Ral isoforms cooperate in the maintenance of oncogenic transformation, mediating both oncogenic proliferation and survival signals (Chien and White, 2003). RalB is specifically required for survival of tumor cells, while RalA is required for anchorage-independent proliferation (Chien and White, 2003). Ral guanine nucleotide exchange factors (GEF) RalGDS, Rgl, Rlf and RGL3 are important downstream effector proteins in Ras signalling pathways (Aguirre-Ghiso *et al.*, 1999; Shao and Andres, 2000). RalGDS cooperates with the Ras-stimulated Raf and PI3K pathways to cause the full Ras-transformed phenotype (Webb *et al.*, 1998). Ral activation is controlled by both Ras-dependent and Ras-independent events. Ras-independent activation of Ral occurs in response to elevated levels of Ca^{2+} (Urano *et al.*, 1996; Hofer *et al.*, 1998; Park, 2001) which allows Ral to participate in Ca^{2+}-dependent intracellular signalling pathways (Wang *et al.*, 1997). Ca^{2+} may also activate RalA through the binding of CaM. Ca^{2+}/CaM enhanced GTP binding to RalA (Wang *et al.*, 1997; Wang

and Roufogalis, 1999). RalA, but not RalB, contains a putative C-terminal CaM binding domain (BD) that forms a hydrophobic amphiphilic helix (Wang *et al.*, 1997). There is also evidence of cross-talk between signal transduction pathways mediated by Ca^{2+}/CaM and Ras proteins (Suzuki *et al.*, 2000). Ral is also activated via Ras-independent RalGEFs RalGPS (Rebhun *et al.*, 2000) and BCAR3/AND-34 (Gotoh *et al.*, 2000). Murine AND-34 forms a complex in cells with the docking protein $p130^{Cas}$, which leads to inhibition of AND-34 GEF activity (*et al.*, 2000). Both $p130^{Cas}$ and AND-34 may activate common signalling pathways when over-expressed, such as the c-Src pathways. RalGEFs can activate c-Src (Goi *et al.*, 2000), and $p130^{Cas}$ binds to and activates c-Src (Burnham *et al.*, 2000), and therefore participates in c-Src signaling pathways (Hakak and Martin, 1999).

Epidermal growth factor (EGF)-induced mitogenesis depends on c-Src activation (Maa *et al.*, 1995; Schlessinger, 2000), which occurs through the Ras/RalGEF/Ral pathway (Goi *et al.*, 2000). Activated c-Src in turn, leads to activation of STAT3 and subsequent gene transcription (Goi *et al.*, 2000). Ral, EGFR, c-Src and STAT3 are intimately connected in the pathogenesis of cancer. An increasing number of human cancers display over-expressed EGF receptor (EGFR) (Reese and Slamon, 1997) and elevated c-Src and STAT3 activity (Berclaz *et al.*, 2001), and Ral may enhance the oncogenic potential of neoplastic cells containing amplified EGF or ERB2 genes (Goi *et al.*, 2000; Lu *et al.*, 2000). AND-34 and $p130^{Cas}$ regulate hormone-dependent proliferation of breast cancer cells and the induction of tamoxifen resistance, in a Ral-dependent manner (Gotoh *et al.*, 2000).

Ral and RalGDS are involved in cardiac hypetrophy in a STAT3-dependent manner (Kawai *et al.*, 2003). Therefore, one protein that may be regulated by CaM-

activated Ral is the β1 subunit of the Na/K pump, Na^+/K^+-ATPase. Ouabain, phorbol esters and growth factors upregulate β1 subunit mRNA transcription (Bhutada and Ismail-Beigi, 1991; Kometiani *et al.*, 2000). Ouabain-stimulated β1 expression in cardiac myocytes is dependent on protein kinase C (PKC), CaM and Ca^{2+} (Huang *et al.*, 1997a), and is regulated by both Ras/ERK-dependent and –independent pathways (Kometiani *et al.*, 2000). Na^+/K^+-ATPase has two distinct roles in the plasma membrane: one being its classical function as an ion pump (Skou and Esmann, 1992; Lingrel and Kuntzweiler, 1994), and the other as a signal transducer (Liu *et al.*, 2000). Ouabain-induced non-toxic inhibition of Na^+/K^+-ATPase activates multiple interrelated signal transduction pathways, including Ras via c-Src and the EGFR, which activates the Raf/MEK/ERK cascade (Haas *et* al, 2000, 2002) and generates mitochondrial reactive oxygen species (ROS) (Xie *et al.*, 1999; Liu *et al.*, 2000). Both events stimulate cardiac myocyte hypertrophic growth and protein synthesis (Tian *et al.*, 2001; Xie and Askari, 2002). There is cross-talk between the ion pumping functions and the signal-transducing functions of Na^+/K^+-ATPase that may depend on Ca^{2+} (Mohammadi *et al.*, 2001). The aim of the current research project was to examine the role of Ral as a CaM binding protein in signal transduction pathways in human platelets and tissue culture cells.

2. LITERATURE REVIEW

2.1 Ral-GTPase/Calmodulin Interaction

2.1.1 Ral-GTPases

Ral proteins, consisting of Ral A and Ral B (85% identical and differing primarily at the C-terminus), are small GTPases belonging to the Ras superfamily of low molecular weight (20-29 kDa) GTP-binding proteins (Chardin and Tavitian, 1986, 1989) (Fig. 1). Like all GTPases, Ral proteins cycle between the active GTP-bound and inactive GDP-bound states (Feig *et al.*, 1996) (Fig. 2). Ral proteins are directed to membranous fractions of cells by the posttranslational geranylgeranylation of their carboxy (C)-terminal CAAX box (Kinsella *et al.*, 1991). Ral proteins are widely distributed, with highest levels in human platelets (Polakis *et al.*, 1989; Bhullar *et al.*, 1990; Bhullar, 1992), brain and testis (Olofsson *et al.*, 1988; Wildey *et al.*, 1993). They have diverse subcellular localizations, including plasma membrane (Polakis *et al.*, 1989; Feig and Emkey, 1993) and cytoplasmic vesicles, such as endocytic vesicles (Polakis *et al.*, 1989; Feig and Emkey, 1993), clathrin-coated, secretory, synaptic and axonal rapid transport vesicles (Bielinski *et al.*, 1993; Volknandt *et al.*, 1993; Huber *et al.*, 1994). In platelets, Ral proteins are present in the plasma membrane (Polakis *et al.*, 1989) and in dense granules, a class of secretory organelles (Mark *et al.*, 1996).

2.1.2 Ral-GAPs and Ral-GEFs

Ral-specific GTPase activating protein (GAP) activities have been identified in brain, testis and platelets (Emkey *et al.*, 1991; Bhullar and Seneviratne, 1996), and many Ral-specific guanine nucleotide exchange factors (RalGEF) have been isolated, such as

RalA MAANKPKGQNSLALHKVIMVGSGGVGKSALTLQFMYDEFVDYEPTKADSYRK

RalB MAANKSKGQSSLALHKVIMVGSGGVGKSALTLQFMYDEFVEDYEPTKADSYRK

RalA KVVLDGEEVQIDILDTAGQEDYAAIRDNYFRSGEGFLCVFSITEMESFAATADFR

RalB KVVLDGEEVQIDILDTAGQEDYAAIRDNYFRSGEGFLLVFSITEHESFTATAEFR

RalA EQILRVKEDENVPFLLVGNKSDLEDKRQVSVEEAKNRAEQWNVNYVETSAK

RalB EQILRVKAEEDKIPLLVVGNKSDLEERRQVPVEEARSKAEEWGVQYVETSAK

RalA TRANVDKVFFDLMREIRARKMEDKEKNGKKKRKSLAKRI RERCCIL

RalB TRANVDKVFFDLMREIRTKKMSENKDKNGKKSSKNKKSFKERCCLL

Fig. 1. Amino acid sequence of human RalA and RalB. Sequences of RalA and RalB differ primarily in the C-terminal amino acids (*green*). Nucleotide binding regions (*orange*). CAAX motif (*pink*).

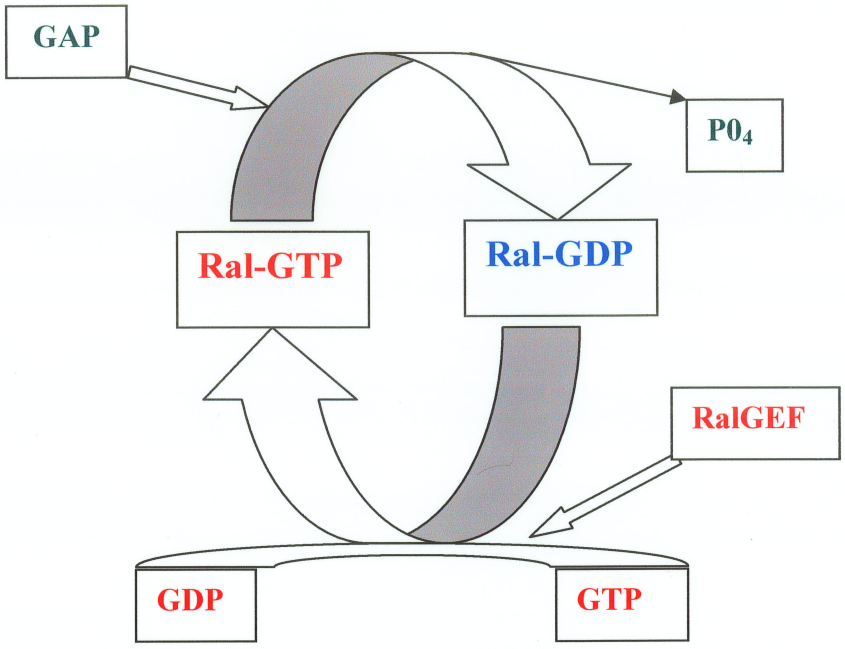


Fig. 2. Ral-GTPase cycle. Ral GTPase activating proteins (GAP) stimulate Ral's intrinsic GTPase activity to hydrolyse active Ral-bound GTP to GDP with loss of the γ-phosphate group. This results in inactive GDP-bound Ral. Upon activation, Ral guanine nucleotide exchange factors (RalGEF) catalyze the replacement of GDP with GTP, resulting in active Ral.

Ral GDP dissociation stimulator (RalGDS), Rgl and Rlf, the oncoprotein Rsc (Albright *et al.*, 1993; Kikuchi *et al.*, 1994; Wolthuis *et al.*, 1996; Peterson *et al.*, 1996; D'Adamo *et al.*, 1997; Murai *et al.*, 1997; Herberg *et al.*, 1998), RalGEFs with pleckstrin homology [PH] domain and Src homology 3 [SH3] binding motif (RalGPS) proteins (Rebhun *et al.*, 2000), RalGEF2 (de Bruyn *et al.*, 2000), breast cancer anti-estrogen receptor locus 3 (BCAR3/AND-34) (Gotoh *et al.*, 2000) and RGL3 (Shao and Andres, 2000).

2.1.3 Involvement of Ras-dependent Ral Activation in Cellular Proliferation and Oncogenesis

Although biochemically well characterized, the functions of Ral-GTPases are largely unknown, and it is not known whether they have non-overlapping, fully over-lapping or partial over-lapping functions (Chien and White, 2003). They are however, involved in signal transduction pathways, including the control of cell proliferation, where activation of Ral is required for full Ras-induced oncogenic growth and morphologic transformation (Song *et al.*, 1994; Urano *et al.*, 1996; White *et al.*, 1996; Feig *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997; Aguirre Ghiso *et al.*, 1999; Reuther and Der, 2000) and induction of DNA synthesis (Miller *et al.*, 1997; Rosario *et al.*, 2001). RalA is dispensable for proliferation of human epithelial cells and tumor-derived cell lines, but is required for anchorage-independent proliferation of transformed cells (Chien and White, 2003). RalB is required to prevent transformed cells from initiating programmed cell death. Therefore, Ral isoforms collaborate in the maintenance of oncogenic transformation, mediating both oncogenic proliferation and survival signals (Chien and White, 2003). As well, inhibition of RalA can relieve the sensitivity of tumor

cells to loss of RalB. It relieves mitogenic pressure, partially reversing oncogenic transformation, and therefore reducing tumor cell dependancy on RalB survival pathways (Chien and White, 2003). Because oncogenic transformation minimally requires the acquisition of enhanced proliferative ability, together with suppression of apoptosis (Evan and Vousden, 2001; Green and Evan, 2002), RalGTPases are crucial components of oncogenic regulatory pathways (Chien and White, 2003).

RalGDS, RGL3, Rgl and Rlf contain a C-terminally located Ras binding domain and are thus important downstream effector proteins in Ras signalling pathways (Kikuchi *et al.*, 1994; Hofer *et al.*, 1994; Spaargaren and Bischoff, 1994; McCormick and Wittinghofer., 1996; Urano *et al.*, 1996; Murai *et al.*, 1997; Wolthuis *et al.*, 1997, 1998a; Wolthuis and Bos, 1999; Aguirre-Ghiso *et al.*, 1999; Shao and Andres, 2000). Inhibition of Ral-GEF was shown to suppress certain aspects of oncogenic transformation by Ras (Urano *et al.*, 1996). A dominant negative Ral blocked the tumorigenesis of v-Src- and v-Ras-transformed NIH3T3 cells (Okazaki *et al.*, 1997; Murai *et al.*, 1997; Wolthuis *et al.*, 1997; Aguirre-Ghiso *et al.*, 1999). The Ral pathway is required for the haematogenous metastatic nodule formation by Ras(12V, 37G)-transformed NIH3T3 cells, and extracellular-regulated kinase (ERK) and Ral pathways synergize to produce an activity that stimulates extracellular matrix invasion (Ward *et al.*, 2001). Insulin- and epidermal growth factor (EGF)-induced activation of Ral is inhibited by a dominant negative Ras, demonstrating that mitogen-dependent activation of Ral requires Ras activation (Wolthuis *et al.*, 1998a). Ras-GTP binding activates Ral-GEFs (Urano *et al.*, 1996; Murai *et al.*, 1997; Wolthuis *et al.*, 1997) by targeting them to the Ral-GTPases in the plasma membrane (Kishida *et al.*, 1997).

2.1.4 Coordinate Activation of Raf/ERK, PI3K and RalGEF/Ral/RLIP76 Pathways

Ras-dependent stimulation of Ral, and the subsequent Ral-GEF-enhanced cell proliferation and cellular transformation, function parallel to the Ras-mitogen-activating protein kinase (MAPK or ERK) pathway in several types of cells (Jiang *et al.*, 1995a; Koyama *et al.*, 1996; Urano *et al.*, 1996; White *et al.*, 1996; Miller *et al.*, 1997; Murai *et al.*, 1997; Wolthuis *et al.*, 1997, 1998a; Goi *et al.*, 1999). Ras transformation may be mediated by the coordinate activation of (i) the Raf (i.e. A-Raf, B-Raf, and Raf-1)/ERK pathway that stimulates mitogenesis and plays a major role in cell differentiation and proliferation (Lewis *et al.*, 1998; Robinson and Cobb, 1997; Marshall, 1999), (ii) the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which is involved in enhancing cell survival, as well as cell proliferation and metabolism (Rodriguez-Viciana *et al.*, 1994; Coffer *et al.*, 1998; Alessi and Cohen, 1998), and (iii) the RalGEF pathway (i.e. RalGDS, Rlf and Rgl), which activates Ral binding protein, RLIP76, that in turn induces cytoskeletal reorganization, phospholipase D1 (PLD1) activation, c-fos and cyclin D1 induction, which are required for mitogenesis (Vojtek and Der, 1998; Wolthuis and Bos, 1999; Reuther and Der, 2000). RalGDS contributes to Ras transformation and cooperates with the Raf and PI3K pathways to cause the full Ras-transformed phenotype (Webb *et al.*, 1998). RalGDS and Raf synergistically stimulate cellular proliferation and gene expression through the c-*fos* promotor (White *et al.*, 1996; Okazaki *et al.*, 1997).

Pathways from Ras to Ral through RalGEFs may be selectively regulated by other Ras-like GTPases such as Rap1 (Ikeda *et al.*, 1995; Kikuchi and Williams, 1996), TC21 (Rosario *et al.*, 2001) and Rit (Shao and Andres, 2000). RalGEFs may be shared between Ras and Rap1 (Bos, 1998). RalGDS, Rgl and Rlf associate with Rap1-GTP *in vitro* and *in*

vivo (Spaargaren and Bischoff, 1994; Wolthuis *et al.*, 1996, Kishida *et al.*, 1997). In platelets, Rap1A functions upstream of Ral, and Ral was stimulated in a fashion similar to Rap1A by platelet agonists α-thrombin and platelet activating factor (Wolthuis *et al.*, 1998b). Elevated levels of calcium (Ca^{2+}) were necessary and sufficient in platelets for the rapid activation of both Ral and Rap1A (Franke *et al.*, 1997; Wolthuis *et al.*, 1998b), and this pathway was inhibited by prostaglandin I_2 (Wolthuis *et al.*, 1998b). Therefore, various findings suggest that Rap1 is able to activate the RalGEF-Ral pathway (Zwartkruis, 1998; Bos, 1998).

RalGDS also binds to R-Ras and TC21 (R-Ras-2) (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994; Spaargaren and Bischoff, 1994; Lopez-Barahona *et al.*, 1996). TC21 directly interacts with RalGEFs, resulting in their translocation to the plasma membrane (Rosario *et al.*, 2001). Apart from the classical Ras proteins (H-Ras, N-Ras, K-Ras-A, K-Ras-B), TC21 is the only other member of the Ras family found to be mutated in human cancers (Chan *et al.*, 1994; Huang *et al.*, 1995; Barker and Crompton, 1998). Ral proteins are involved in cell adhesion, in part, via R-Ras-mediated pathways (Osada *et al.*, 1999). Effector pathways identified that are required for TC21-induced cellular transformations are the Raf/MAPK, PI3K and Ral/RalGEF signalling pathways (Rosario *et al.*, 2001). Constitutively active TC21 stimulated RalA and the PI3K-dependent activation of Akt/PKB. Activation of the Ral pathway by TC21 was shown to be necessary for TC21-stimulated DNA synthesis, and the Ral/TC21 pathway was shown to be required for the proliferation of human tumour cells. Thus, the Ral pathway is involved in the stimulation of DNA synthesis in human tumour cell lines (Rosario *et al.*, 2001).

2.1.5 Role of Ras in Transformation of Human and Murine Cells

Activation of RalGEF signalling effectors is sufficient to cause growth transformation in a variety of human cells, but not in rodent cells, and activation of RalGEFs is essential for Ras transformation in human cells (Hamad *et al.*, 2002). Activation of neither the Raf/ERK (in contrast to murine fibroblasts [Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997]) nor PI3K pathways alone is sufficient to transform human HEK-HT epithelial cells. While the coordinate activation of the Raf and PI3K pathways of oncogenic Ras works synergistically to transform murine NIH3T3 cells (Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997), it fails to transform HEK-HT cells (Hamad *et al.*, 2002). However, they do act together to enhance transformation induced by the RalGEF pathway (Hamad *et al.*, 2002). Therefore, in mice, Ras sends its oncogenic signal primarily through Raf, while in human cells, the oncogene appears to depend mainly on RalGEF effector branch for its transforming ability (Boettner and Van Aelst, 2002). It is not known how RalGEFs transform human cells and whether RalGEFs are upregulated in human tumors that harbour mutated Ras.

2.1.6 Possible Mechanisms by which RalGEFs Promote Tumour Formation Downstream of Ras

2.1.6.1 Regulation of cell cycle

There is sparse evidence for the involvement of Ral in the cell cycle machinery. An activated mutant RalGEF, Rlf-CAAX, is involved in cyclin D1 transcription and E2F activity, two principal agents of G1/S progression in human cells (Gille and Downward, 1999; Wolthuis and Bos, 1999). Ral also induces cyclin D1 transcription, in an NF-κB-

dependent manner (Henry *et al.*, 2000). A dominant-negative Ral interfered with the ability of human fibrosarcoma HT1080 cells, which contain an activating mutation in their N-Ras gene, to grow anchorage-independently in soft agar (Yamazaki *et al.*, 2001). Activated Ral decreased the level of $p27^{Kip1}$, an inhibitor of cell cycle promoting cyclin/CDK complexes. The opposite effect was observed with the dominant-negative Ral (Yamazaki *et al.*, 2001). In the human DLD1 colon carcinoma cell line, a Ras/Ral-specific pathway leads to the phosphorylation of the forkhead transcription factor AFX at two critical residues (de Ruiter *et al.*, 2001). This modification represses the transcriptional activity of AFX to decrease the level of $p27^{Kip1}$, consequently alleviating G1 progression of the cell cycle. Ral, by virtue of its Ral binding partner RLIP76/RalBP1, modulates the internalization of growth factor-dependent receptors, such as epidermal growth factor receptor (EGFR) and insulin receptor (Nakashima *et al.*, 1999; Jullien-Flores *et al.*, 2000). Both constitutively active and dominant-negative mutants of Ral block EGF uptake in human A431 epithelial carcinoma and other cells (Nakashima *et al.*, 1999). This suggests that Ral needs to cycle between its GDP- and GTP-bound forms under normal homeostatic circumstances and that its overactivation by oncogenic Ras could shift the balance to allow sustained activity of EGF receptors. EGFR engagement stimulates Src kinase with an impact on its own internalization (Wilde *et al.*, 1999). EGF-dependent Src activation depends on Ral activity (Goi *et al.*, 2000). Interference with growth factor receptor internalization by Ral may be generalized to other cancer-relevant receptor tyrosine kinase systems (Boettner and Van Aelst, 2002).

2.1.6.2 Ral activates PLD1 in a Src-dependent manner

Ral proteins as Ras effectors may regulate phospholipid metabolism because RalA is involved in the tyrosine kinase-mediated activation of PLD, perhaps through a direct, constitutive association via Ral's unique N terminus with PLD1 (Jiang *et al.*, 1995a; Urano *et al.*, 1996; Luo *et al.*, 1997, 1998; Schmidt *et al.*, 1998; Kim *et al.*, 1998). In platelets and other cells, PLD has been implicated in vesicle transport and secretion, regulation of the actin cytoskeleton, and generation of lysophosphatidic acid, which is secreted by platelets upon activation (Knight *et al.*, 1984; Haslam and Coorssen, 1993; Coorssen, 1996; Coorssen *et al.*, 1996; Exton, 1997; Morgan *et al.*, 1997; Moolenaar *et al.*, 1997). RalA was necessary but not sufficient for the activation of PLD in v-Src-transformed NIH3T3 cells (Jiang *et al.*, 1995a). Both Ral and Arf are needed for PLD activation (Luo *et al.*, 1998; Kim *et al.*, 1998). The v-Src-induced PLD activity is dependent on Ras (Jiang *et al.*, 1995b), and Ras induces an increase in PLD activity (Carnero *et al.*, 1994). A general model (Luo *et al.*, 1998) has been proposed in which Ras is activated by v-Src, which causes RalGDS to be translocated to the plasma membrane. RalGDS then brings RalA and constitutively-associated PLD1 into a tyrosine kinase/Ras signalling complex. Ral is activated, which in turn, with the help of Arf-GTP, and perhaps Rho and protein kinase C (PKC), activates PLD to generate lipid second messengers (Jiang *et al.*, 1995a; Exton, 1997; Wolthuis *et al.*, 1998b; Goi *et al.*, 1999).

2.1.6.3 Ral/PLD and EGFR

Via its multiple lipid second messengers, PLD probably contributes significantly to cellular responses activated by Src and mediated by Ras and its effector, RalGEF. PLD may be one agent that allows Ral to enhance both Ras- and Raf-induced cellular

transformation (Jiang *et al.*, 1995a; Urano *et al.*, 1996). In 3Y1 rat fibroblasts, over-expressing the EGFR induced Ras-dependent activation of Ral, and both Ras- and Ral-dependent activation of PLD (Lu *et al.*, 2000). RalA is required for the EGF-induced activation of PLD (Lu *et al.*, 2000). The transformed phenotype induced by EGF in cells overexpressing the EGFR was also dependent upon RalA, suggesting PLD is an important component of the EGF-induced cell division signals. Overexpression of either RalA or PLD1 in EGFR cells led to anchorage-independent growth in the absence of EGF. Therefore, either RalA or PLD1 can cooperate with the EGFR to transform cells (Lu *et al.*, 2000). The overexpressed EGFR induces only a partially transformed phenotype, and RalA is essential for mitogenic signalling mediated by an activated EGFR (Lu *et al.*, 2000).

2.1.6.4 Ral/PLD and caveolae

Many signalling molecules, including Ral, Ras, Src and EGFR (Anderson, 1998; Lu *et al.*, 2000), as well as PLD1 and PLD2 (Czarny *et al.*, 1999; Kim *et al.*, 1999), localize to caveolin-enriched light membrane fractions. PLD may somehow recognize signalling molecules in this plasma membrane microdomain, and contribute to the formation of "signaling vesicles" that are endocytosed from the plasma membrane (Luo *et al.*, 1998). RalA is required for EGF-induced receptor-mediated endocytosis (Nakashima *et al.*, 1999) and this step is required for many of the signals generated in response to EGF (Vieira *et al.*, 1996). Therefore, PLD and Ral may be involved in the generation of endocytic signaling vesicles (Lu *et al.*, 2000).

2.1.6.5 Ral/PLD pathway and proteases

The RalA/PLD pathway may be connected to the involvement of proteases in tumorigenicity and metastasis (Aguirre-Ghiso *et al.*, 1999). Proteases like urokinase-type plasminogen activator (uPA) and matrix metalloproteases (MMP) are intimately involved in metastasis (Liotta and Stetler-Stevenson, 1991; Ossowski, 1992), and such proteases are upregulated upon transformation by v-Src and v-Ras (Sato *et al.* 1993; Bell *et al.*, 1993; Gum *et al.*, 1997; Silberman *et al.*, 1997; Aguirre-Ghiso *et al.*, 1999). PLD activity is increased in response to most if not all mitogenic signals (Foster and Xu, 2003). uPA production is upregulated in v-Src- and v-Ras-transformed NIH3T3 cells in a RalA-mediated manner, and MMPs were upregulated by v-Src in a RalA-dependent manner (Aguirre-Ghiso *et al.*, 1999). These data implicate RalA and PLD as signalling mediators for tumour formation and protease production by transformed cells (Aguirre-Ghiso *et al.* (1999).

2.1.6.6 Ral stimulates uPA receptor transcription in a Src-dependent manner

The uPA receptor (uPAR) plays an important role in determining malignancy of most human tumours (Andreasen *et al.*, 1997, 2000; Okan *et al.*, 2001). Transcription of uPAR was stimulated by active Ras and Ral mutants (Okan *et al.*, 2001). RalA-dependent uPAR transcription requires c-Src, identifying c-Src as a downstream effector in this RalA signalling pathway (Okan *et al.*, 2001). The results also suggest that Ral proteins may play an important role in the development of cancer, perhaps by affecting the invasive and metastatic properties of transformed cells (Reuther and Der, 2000). Ras and RalGEF mutants in NIH3T3 cells revealed an essential interaction of the RalGEF and ERK pathways to produce a malignant phenotype (Ward *et al.*, 2001). The ERK and RalGEF

pathways interact to produce a genetic program leading to a metastatic phenotype by coordinately regulating an essential gene(s) and/or by regulating distinct sets of genes that contribute different features to the metastatic phenotype. The RalGEF pathway significantly contributes to Ras-initiated metastasis (Ward *et al.*, 2001).

2.1.6.7 Ral regulates fibronectin and CD44 expression

There is a correlation between fibronectin (FN) downregulation and increased malignancy of tumour cells (Werbajh *et al.*, 1998), and an upregulation of CD44, a hyaluronan receptor, in v-Src and v-Ras-transformed epithelial cells (Jamal *et al.*, 1994). RalA regulates tumour formation in transformed NIH3T3 cells, mediating signal transduction pathways activated by v-Src, v-Ras and v-Raf, which leads to FN down-regulation and CD44 overexpression (Ladeda *et al.*, 2001), in addition to regulation of proteases (uPA and MMPs) (Aghirre-Ghiso *et al.*, 1999).

2.1.6.8 Ral regulates EGF-mediated cell motility

In human tumour cell lines, EGF has been shown to stimulate motility, which is one of the rate limiting steps of invasion, and which defines progression towards a more malignant phenotype (Gildea *et al.*, 2002). RalA has an essential role in EGF-mediated cell motility, and potentially contributes to tumour metastasis in human cancer (Gildea *et al.*, 2002).

2.1.7 Ras-Independent RalGEFs

The RalGEFs of the RalGPS family and BCAR3/AND-34 do not contain a Ras binding domain, suggesting they are activated in a Ras-independent manner (Rebhun *et al.*, 2000; Gotoh *et al.*, 2000). RalGPS is composed of RalGPS 1A, 1B, and 2. The PH

domain was required for *in vivo* GEF activity (possibly for membrane targeting), and the SH3 domain contributed to RalGPS regulation. This domain bound to the SH3 domain-containing adapter proteins, Grb2 and Nck, and results showed that growth factor stimulation may be responsible for promoting the translocation and/or activation of RalGPS in a Ras-independent manner (Rebhun *et al.*, 2000). RalGEF2 also contains a PH domain which has been shown to function as a membrane anchor necessary for optimal activity *in vivo* (de Bruyn *et al.*, 2000).

Murine AND-34 (human homolog is BCAR3/NSP2) forms a complex in cells with $p130^{Cas}$ (Crk-associated substrate, human homolog is BCAR1), a known c-Src substrate and a docking protein for the v-Src sarcoma virus CT10 oncogene homolog (Crk) adapter protein, focal adhesion kinase Pyk-2, and PTP-PEST phosphatases (Sakai *et al.*, 1994). AND-34 is an activator of Ral, and to a lesser extent R-Ras and Rap1A. $p130^{Cas}$ inhibits the GEF activity of AND-34, possibly by steric hindrance of GTPase binding to the catalytic domain (Gotoh *et al.*, 2000). Both $p130^{Cas}$ and AND-34 may activate common signalling pathways when overexpressed such as the c-Src pathways. RalGEFs can activate c-Src, and $p130^{Cas}$ binds to and activates c-Src and therefore c-Src signalling pathways (Hakak and Martin, 1999; Burnham *et al.*, 2000). Of possible relevance, both $p130^{Cas}$ and AND-34 are implicated in the resistance of breast cancer cells to the anti-estrogen tamoxifen (van Agthoven *et al.*, 1998; Brinkman *et al.*, 2000; Van der Flier *et al.*, 2000), which depends on Ral, Rap1 and R-Ras. AND-34 and $p130^{Cas}$ function in a common signalling pathway to regulate hormone-dependent proliferation of breast cancer cells (Gotoh *et al.*, 2000). AND-34 expression correlates with loss of estrogen receptors (ER) in breast cancer cell lines (van Agthoven *et al.*, 1998), and high

levels of $p130^{Cas}$ in primary human breast carcinomas are associated with poor relapse-free survival, poor overall survival, and a reduced response rate to tamoxifen in patients with recurrent disease (Van der Flier *et al.*, 2000). This suggests that AND-34 functions through $p130^{Cas}$ in the induction of tamoxifen resistance (Gotoh *et al.*, 2000).

A critical step in the progression of breast cancer occurs when tumor cells no longer rely on estrogen to proliferate, and are therefore no longer responsive to estrogen antagonists such as tamoxifen (Yu and Feig, 2002). Although excess Ral activity alone is not sufficient to induce estrogen-independent proliferation in MCF-7 cells, Ral activation is important for EGF receptors to induce estrogen-independent proliferation of these cells (Yu and Feig, 2002). Many estrogen-insensitive tumors over-express growth factor receptors such as EGFR (HER1) and HER2 (Yu and Feig, 2002).

2.1.8 Ral Binding Proteins

The family of nearly identical Ral effector proteins called Ral interacting protein 1 from mouse, (RIP1) (Park and Weinberg, 1995), 76 kDa Ral interacting protein from human (RLIP76) (Jullien-Flores *et al.*, 1995), and Ral binding protein-1 (RalBP1) from rat (Cantor *et al.*, 1995), contain a GAP region similar to RhoGAP domains, and has GAP activity acting upon CDC42 and Rac. RalBP1 has a Ral binding domain in its C-terminal region and binds to the GTP-bound but not the GDP-bound form of Ral (Feig *et al.*, 1996). The Rho pathway comprises a cascade of GTPases from Cdc42 to Rho passing by Rac, and acts upon structures involved in cell shape plasticity. Therefore, Ral may be involved in the negative regulation of CDC42 and Rac1 (Jullien-Flores *et al.*, 1995; Cantor *et al.*, 1995; Park and Weinberg, 1995), proteins involved in the organization of

the actin cytoskeleton and the regulation of cytoskeletal polarity (Wolthuis *et al.*, 1998b). Activation of Ras leads to several cytoplasmic and nuclear phenomena as well as membrane modifications. This and the fact that Ras can activate Ral-GTPases has led to the proposal that RLIP76 (and other Ral binding proteins) participates in the cross-talk between Ras, Ral and Rho cascades, modulating the state of activity of the Cdc42/Rac/Rho pathway in response to Ras activation (Jullien-Flores *et al.*, 1995). Because RalBP1 has a GAP domain for Rac1 and Cdc42, and PLD activation leads to actin stress fibre formation (Cross *et al.*, 1996), Ral may modulate actin cytoskeletal dynamics by controlling the activities of RalBP1 and PLD (Suzuki *et al.*, 2000).

2.1.8.1 Ral binding proteins and endocytosis

RalBP1 also regulates endocytosis of EGF and insulin receptors (Nakashima *et al.*, 1999). RalBP1 binds to two highly related epsin homology (EH) domain proteins, REPS1 (RalBP1-associated Eps-homology domain protein) and POB1 (partner of RalBP1) (Yamaguchi *et al.*, 1997; Ikeda *et al.*, 1998). POB1 is a binding protein of RalBP1 (Ikeda *et al.*, 1998), and has a single EH domain in its N-terminal region (Nakashima *et al.*, 1999). Both epsin15 and epsin bind directly to the EH domain of POB1 (Nakashima *et al.*, 1999; Morinaka *et al.*, 1999). Eps15 is a substrate of EGFR kinase and is constitutively associated with the plasma membrane and clathrin adaptor protein complex-2 (AP2) (Fazioli *et al.*, 1993; Di Fiore *et al.*, 1997). In endocytic clathrin-coated pits, AP2 complexes function to anchor clathrin triskelions to the membrane and to initiate formation of the clathrin lattice (Schmid *et al.*, 1998). AP2 also concentrates various cargo proteins in coated pits (Jullien-Flores *et al.*, 2000). Membrane-bound Eps15 is associated mainly with clathrin-coated pits and vesicles.

Eps15 is involved in receptor-mediated endocytosis because in CV-1, COS and HeLa cells the AP2 binding region of Eps15 inhibited internalization of the EGF and transferrin receptors (Carbone *et al.*, 1997). The EH domain and the C-terminal region of POB1 blocked the internalization of EGF and insulin, respectively. POB1 directly binds to Grb2 but not to Nck or Crk. EGF induces tyrosine phosphorylation of POB1 and leads to formation of a complex between EGFR and POB1. This suggests RalBP1 and POB1 are involved in EGF signalling (Nakashima *et al.*, 1999). REPS1 also binds to the adaptor proteins Crk and Grb2 (Yamaguchi *et al.*, 1997).

Therefore, a Ral-RalBP1-POB1 complex transmits signals from receptors to epsin and Eps15, thereby regulating ligand-dependent, receptor-mediated endocytosis (Nakashima *et al.* 1999). In a model proposed by Nakashima *et al.* (1999) (Fig. 3), Ras is activated by EGF or insulin, which recruits RalGDS to the plasma membrane. RalGDS stimulates GDP-GTP exchange on Ral, which induces the translocation of RalBP1 complexed with POB1 to the plasma membrane. POB1 associates with Eps15 and epsin, which bind to the AP2-clathrin complex, resulting in the formation of clathrin-coated vesicles with transmembrane receptors. Thus Ral may regulate clathrin-dependent endocytosis (Kariya *et al.*, 2000).

RLIP76 may be one factor responsible for the specificity of the interaction between AP2 and receptors endocytosed in clathrin-coated pits, because the N-terminus of RLIP76 binds specifically to μ2, the median chain of AP2 (Jullien-Flores *et al.*, 2000). μ2/RLIP76 interactions may modulate the ability of μ2 to bind such receptors, thus regulating membrane association of AP2 and formation of coated pits. Thus, Ral-GTP-

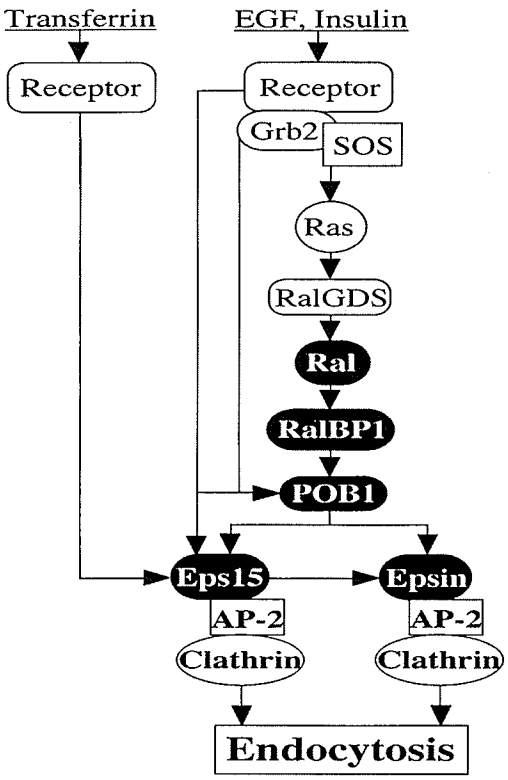


Fig. 3. Ral signaling pathway in the regulation of endocytosis of the EGF and insulin receptors. When Ras is activated by EGF or insulin through Grb2 and SOS, it recruits RalGDS to the plasma membrane, resulting in the activation of Ral. The activated Ral interacts with RalBP1, which forms a complex with POB1. POB1 associates with epsin and Eps15, which bind to the AP-2 and clathrin complex. Grb2 directly binds to POB1. EGF induces tyrosine phosphorylation of Eps15 and POB1 (Nakashima *et al.*, 1999).

dependent recruitment of RLIP76 to the membrane may facilitate receptor interaction with AP2 (Jullien-Flores *et al.*, 2000). When signalling receptors (e.g. EGFR) are stimulated, the activation of Ral would result in additional local recruitment of AP2 and formation of new coated pits. Sequential cycles of activation and recruitment of GTPases and their partners would lead to activated receptors engulfed in coated pits. In that other partners of Ral and RLIP76 are probably involved in AP2 dynamics, it is proposed that RLIP76 must bind to partners of its N-terminal domain (e.g. AP2) and its C-terminal domain (e.g. POB1 and/or REPS1) to behave properly in endocytosis (Jullien-Flores *et al.*, 2000).

Because endocytosis (e.g. of integrins) is an essential process in cell motility (Lawson and Maxfield, 1995), and PLD is involved in membrane trafficking and vesicle transport (Jones *et al.*, 1999), Ral may stimulate cell motility by recycling membrane proteins, including integrins (Suzuki *et al.* 2000). Ral-GTPase interacts directly with filamin-α, an actin cross-linking protein (Gorlin *et al.*, 1990; Takafuta *et al.*, 1998; Xie *et al.*, 1998). Ral may also utilize filamin for cell migration because filamin is a downstream intermediate in Cdc42-mediated filopodia formation. Ral induces the generation of filapodia-like structures, possibly by recruiting filamin into the filapodial cytoskeleton (Ohta *et al.*, 1999).

2.1.9 Ral and Exocytosis

Activated Ral may also play a central role in directing sites of exocytosis, because eight specific proteins that comprise the mammalian exocyst complex associate with RalA in a GTP-dependent manner in GST-RalA pull-down experiments and matrix-

assisted laser desorption ionisation time-of-flight mass spectrometry analysis in rat brain (Brymora *et al.*, 2001). Thus, RalA may regulate both endocytosis and exocytosis at nerve terminals. Exocytosis and endocytosis in nerve terminals is Ca^{2+}-dependent, and RalA is also activated *in vitro* by the Ca^{2+}/calmodulin (CaM) complex (Wang and Roufogalis, 1999; Park, 2001). The exocyst complex is required for exocytosis and neurite outgrowth, and it localizes to filapodia and neurite growth cones. Therefore, RalA may regulate the integration of receptor and Ca^{2+} signalling with neurite outgrowth, endocytosis, and direct sites of exocytosis (Brymora *et al.*, 2001).

2.1.10 Ras-Independent Activation of Ral by Calcium

Activation of Ras does not always lead to Ral activation, since in neutrophils granulocyte-macrophage colony-stimulating factor induced the activation of Ras but not Ral, suggesting that Ral is controlled by both Ras-dependent and Ras-independent events (Bos, 1998). Ras-independent activation of Ral does occur in response to elevated levels of Ca^{2+} (Urano *et al.*, 1996; Hofer *et al.*, 1998; Wolthuis *et al.*, 1998b; Zwartkruis *et al.*, 1998; M'Rabet *et al.*, 1999; Wang and Roufogalis, 1999), and it has been postulated that RalA is associated with Ca^{2+}-dependent intracellular signalling pathways (Wang *et al.*, 1997). The platelet agonists platelet activating factor, thromboxane A_2 and α-thrombin all activated RalA in platelets, suggesting that RalA activation is mediated by a common signalling event (Wolthuis *et al.*, 1998b). This event may involve Ca^{2+}. The α-thrombin-mediated activation of Ral in platelets was inhibited by BAPTA-AM-mediated depletion of, but stimulated by the thapsigargin- or ionomycin-mediated increase in, intracellular Ca^{2+}, and increased levels of intracellular Ca^{2+} were sufficient for Ral activation

(Wolthuis *et al.*, 1998b). Ca^{2+} stimulates the association of [γ-^{35}S]GTP with RalA and RalB in a dose-dependent manner (Park, 2001). Ral activation by lysophosphatidic acid or EGF can be blocked by a phospholipase C (PLC) inhibitor (Hofer *et al.*, 1998). It is known that the activation of platelets by platelet agonists requires activation of the heterotrimeric G protein G_q (Offermans *et al.*, 1997), which mediates the activation of PLCβ (Neer, 1995; Offermans *et al.*, 1997). PLCβ hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2), releasing the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 stimulates the release of Ca^{2+} from internal stores, which may trigger the influx of Ca^{2+} (Schlessinger, 2000; Prenzel *et al.*, 2001). Of relevance to exocytosis in platelets, Ral is activated by agents that stimulate secretion in platelets. For example, α-thrombin induces the fusion of dense granules with the plasma membrane and the open canalicular system (Morgenstern, 1995).

2.1.11 Ca^{2+}-binding Protein Calmodulin Activates Ral

Exactly how Ca^{2+} activates RalA is unclear. Phosphorylation by Ca^{2+}-dependent kinases may regulate the activity of RalGEFs and RalGAPs, or there may be Ca^{2+}-dependent RalGEFs (Wolthuis *et al.*, 1998b). Calcium may also activate RalA through the binding of CaM to a region in the carboxy terminus of RalA (Wang *et al.*, 1997; Wang and Roufogalis, 1999). CaM acts as a cellular Ca^{2+} sensor for many enzymes; regulates ion channels, the cell cycle, and cytoskeletal organization; and influences development (Heiman *et al.*, 1996). It acts as a second messenger in cellular signal transduction pathways and regulates cell proliferation (Klee and Vanaman, 1982; Lu and Means, 1993; Shulman, 1993; Herget *et al.*, 1994), gene expression, protein translation

and protein phosphorylation (Villalonga *et al.*, 2001). There is a 100% identity in its amino acid residues among vertebrates, with multiple genes encoding identical CaMs (Friedberg, 1990). It is a small (148 amino acids), relatively acidic protein, that undergoes conformation change upon Ca^{2+} binding, and that has four E-F hands (Chin and Means, 2000). This structural motif consisits of an N-terminal helix (E helix), immediately followed by a centrally located Ca^{2+} coordinating loop, and a C-terminal helix (F helix). The first two E-F hands combine to form a globular N-terminal domain that is separated by a short flexible linker from a highly homologous C-terminal domain consisting of E-F hands 3 and 4 (Chin and Means, 2000). CaM functions are mediated by its association with specific target proteins called CaM binding proteins (CaMBPs) whose activity is regulated upon CaM binding (Bachs *et al.*, 1994; Agell *et al.*, 1998). Depending on the specific protein, CaM binds CaMBPs in the presence of Ca^{2+} (Ca^{2+}/CaM) or in its absence (apoCaM) (Jurado *et al.*, 1999). CaM binding regions of target proteins show no strong sequence homology, but are characterized by a basic amphipathic α-helix consisting of approximately twenty amino acid residues (Persichini and Kretsinger, 1988; O'Neil and DeGrado, 1990) that have a net positive charge, moderate hydrophilicity, and moderate to high helical hydrophobic moment (O'Neil and DeGrado, 1990).

Ral appears to be a CaM binding protein. *In vitro* experiments with endogenous erythrocyte membrane and recombinant RalA proteins demonstrated that RalA bound CaM in a Ca^{2+}-dependent manner (Wang *et al.*, 1997). As well, Ca^{2+}/CaM dissociated RalA from synaptic vesicle membranes in an apparent Ca^{2+}-dependent manner (Park *et al.*, 1999; Park, 2001). This suggests that the cycling of RalA by CaM is dependent on the presence of Ca^{2+}, while the GDP/GTP-binding state of RalA appeared to only mildly

affect the dissociation of RalA by Ca^{2+}/CaM. Only Ca^{2+}-activated CaM dissociates RalA from membranes by perhaps forming a 1:1 complex, and Ca^{2+}/CaM interacts directly with RalA regardless of GTP or GDP (Park, 2001). RalA protein is found exclusively in membranous fractions of cells (Feig *et al.*, 1996). Although it is not known how RalA translocates from the cytosol to the membranes, it is speculated that CaM may lose the binding capacity with RalA by lowering the Ca^{2+} concentration. This releases RalA from the RalA/Ca^{2+}/CaM complex, allowing it to translocate to membranes, which may be essential for the regulation of synaptic vesicles (Park *et al.*, 1999). It is proposed that the disappearance of RalA from synaptic vesicles causes termination of the signal pathway through RalA. Alternatively, the RalA dissociated by Ca^{2+}/CaM may be activated through GTP binding, with subsequent activation of Ral effectors (Park *et al.*, 1999).

Ca^{2+}/CaM enhanced by a factor of three the GTP binding to RalA isolated from CaM-depleted erythrocyte membranes in a Ca^{2+}-dependent manner (Wang *et al.*, 1997; Wang and Roufogalis, 1999). Ca^{2+} alone did not affect GTP binding to RalA, indicating that the GTP binding is not directly enhanced by Ca^{2+}, but requires CaM in its Ca^{2+}-bound form (Wang and Roufogalis, 1999). As well, endogenous levels of activated GTP-bound RalA were increased by treatment with ionomycin in rat fibroblasts (Hofer *et al.*, 1998). Although Ca^{2+}/CaM, but not Ca^{2+} or CaM alone, stimulates the GTPase activity of RalA, it does not change the GTP- or GDP-binding to RalA, but may stimulate the rate of GTP/GDP turnover (Park, 2001). It is proposed that the Ca^{2+}-dependent CaM binding to RalA may accelerate the displacement of bound GDP, promoting GTP binding. Thus, CaM may be a potential effector for Ral-GTPase activation and serve as a molecular switch in response to changes in intracellular Ca^{2+} concentration over the physiological

range. It would serve this role as a minor player in conjunction with RalGAP and RalGEF regulatory factors (Wang and Roufogalis, 1999). Another mechanism may be that CaM binding to Ral may decrease its GTPase activity. Other suggestions for the Ca^{2+}/CaM-induced enhanced sensitivity of GTP binding to RalA include increase in the number and/or affinity of GTP-binding sites, and displacement by CaM of an autoinhibitory domain from the active site (Vogel, 1994; Wang and Roufogalis, 1999).

RalA, but not RalB, contains a putative C-terminal CaM-binding domain (CaM BD) that is comprised of basic/hydrophobic residues and that readily forms an amphiphilic alpha-helix (Wang *et al.*, 1997). Therefore, Ca^{2+}/CaM activate RalA and the signalling pathways of Ca^{2+}/CaM and RalA are directly linked (Park *et al.*, 1999). There is also the possibility of cross-talk between signal transduction pathways mediated by Ca^{2+}/CaM and Ras proteins (Wang *et al.*, 1997). For example, insulin-like growth factor 1 (IGF-1) utilized both the Ras- and Ca^{2+}-mediated pathways for the coordinate regulation of Ral activity (Suzuki *et al.* 2000).

2.2 Regulation of EGF-Induced Activation of Ral-GTPases

2.2.1 Receptor Tyrosine Kinase Activation of c-Src and its Substrates

Receptor and cytoplasmic protein tyrosine kinases are key players in the control of many cellular processes, including embryonic development and the regulation of many metabolic and physiological processes (Hunter, 2000; Schlessinger, 2000; Pawson *et al.*, 2001). Therefore, aberrations in function or localization of any of the components of these receptor-mediated intracellular signalling pathways will result in severe diseases, such as cancer, diabetes, immune deficiencies and cardiovascular disease (Blume-Jensen

and Hunter, 2001). The EGFR and the non-receptor tyrosine kinase, c-Src, are critical elements in relaying signals from G-protein-coupled receptors (GPCR), cytokines and cellular stress to a variety of cellular responses, including the activation of MAPKs (Luttrell *et al.*, 1999a; Ma *et al.*, 2000; Abram and Courtneidge, 2000; Chen *et al.*, 2001; Andreev *et al.*, 2001; Prenzel *et al.*, 2001). c-Src is activated by many stimuli, including tyrosine kinase receptors, G protein-linked receptors (Luttrell *et al.*, 1997), integrins (Schlaepfer and Hunter, 1997), and estrogen receptors (Migliaccio *et al.*, 1996). Activated c-Src, in turn, promotes the phosphorylation of many proteins, including EGFRs (Biscardi *et al.* 1999a), adapter protein Src homology-containing (Shc), transcription factor signal transducer and activator of transcription-3 (STAT3), and endocytic proteins clathrin (Wilde *et al.* 1999) and dynamin (Ahn *et al.*, 1999). The G protein-linked β_2-adrenergic receptors activate c-Src by promoting its interaction with the adapter protein arrestin (Luttrell *et al.*, 1999b). Activated Src then promotes the phosphorylation of either Shc or GAB1, which leads to the activation of the Ras/MAPK signalling cascade, thus mediating the growth-promoting effects of G protein-linked receptors (Goi *et al.*, 2000).

2.2.2 EGF-induced Activation of c-Src Occurs via Ras/RalGEF/Ral Pathway

EGF binding to its EGFR stimulates tyrosine phosphorylation of cellular substrates, including transphosphorylation of the EGFR itself (Carpenter and Cohen, 1979; Hunter and Cooper, 1985; Schlessinger, 1986, 1988; Yarden and Ulrich, 1988). Subsequent responses lead to the commitment of the cell to enter mitosis, resulting in cell division (Carpenter and Cohen, 1979; Schlessinger, 1986). EGF-induced mitogenesis

depends on non-receptor tyrosine kinase c-Src activity (Maa *et al.*, 1995). c-Src contributes to EGF effects on gene transcription through phosphorylation of STAT transcription factors (Bromberg *et al.*, 1998; Turkson *et al.*, 1998), and on cell shape through cytoskeletal substrates such as cortactin (Belsches *et al.*, 1997). EGF binding to the EGFR activates c-Src through the Ras/RalGEF/Ral pathway (Goi *et al.* 2000) (Fig. 4). Activated c-Src in turn, leads to activation of the transcription factor STAT3 and the actin binding protein cortactin (Goi *et al.* 2000). Although in certain signaling pathways, usually involving transactivation of the EGFR, c-Src can activate ERK by promoting tyrosine phosphorylation of the adapter protein Shc (Luttrell *et al.*, 1997; Schlaepfer and Hunter, 1997), the EGF-activated c-Src can phosphorylate the EGFR itself, but this phosphorylation does not promote the Ras/ERK signaling cascade (Biscardi *et al.*, 1999a; Tice *et al.*, 1999). Also, expression of the constitutively active allele of RalA, Ral72L, did not enhance phosphorylation of Shc in 293 cells, and had no effect on ERK activity. Similarly, the expression of a dominant-negative Ral had no effect on EGF activation of ERK (Goi *et al.*, 2000). However, both a RalGEF (Rgr) and constitutively active RalA (RalA72L) increased tyrosine-phosphorylated STAT3 in PC12 cells, and the IL-6-dependent tyrosine phosphorylation of STAT3 depends on RalGEF (Goi *et al.*, 2000). Overexpression of Rgr, RalA72L or the constitutively active RasH61L in HEK293 cells enhanced c-Src activity by about 2-fold, while EGF treatment increased its activity about 3-fold. Ral activation by RalGEF is also required for EGF-induced c-Src activation in these cells. The dominant-negative Ras17N suppressed EGF activation of c-Src, demonstrating that EGF activates Ral through the activation of Ras. RalA72L promoted

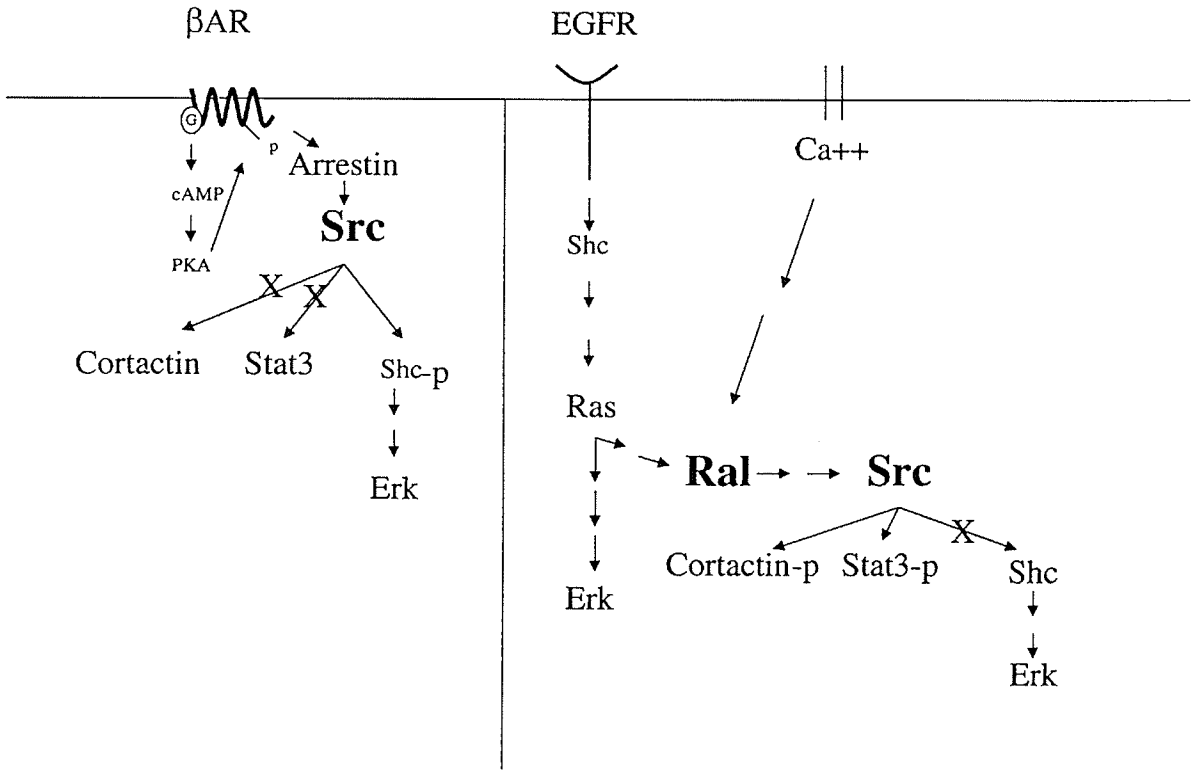


Fig 4. EGF and β-adrenergic receptors activate c-Src by different mechanisms that lead to the tyrosine phosphorylation of different sets of c-Src substrates. EGF receptors, but not β-adrenergic receptors (βAR), activate c-Src by a Ras- and Ral-dependent signaling pathway. Importantly, Ral-induced c-Src activation leads to STAT3 and cortactin tyrosine phosphorylation, but not Shc phosphorylation and ERK activation. In contrast, βAR activation of c-Src leads to Shc but not cortactin or STAT3 tyrosine phosphorylation. Ral can also be activated by Ras-independent pathways, such as through calcium, which would allow c-Src to activate downstream targets without activating Ras and its multiple effectors. Overall, these findings suggest how different receptors may generate specific cellular effects through a common kinase (Goi *et al.*, 2000).

association of c-Src with STAT3 to a level equivalent to that seen with EGF, and induced tyrosine phosphorylation of endogenous STAT3 and cortactin. These effects were suppressed by a dominant-negative Ral (Goi *et al.*, 2000). Therefore, Ral- or EGF-activated c-Src leads to tyrosine phosphorylation of cortactin or STAT3 by Ras- and Ral-dependent pathways, but not Shc phosphorylation and ERK activation. In contrast, isoproterenol induced the GPCR activation of Shc and ERK, but not STAT3 or cortactin (Goi *et al.*, 2000). The Ral-dependent coupling of EGF to transcription through STAT3 and to the actin cytoskeleton through cortactin may also occur by a Ras-independent pathway, such as through Ca^{2+}. This would allow c-Src to activate downstream targets without activating Ras and its effectors (Goi *et al.*, 2000). Therefore, EGF/Ral and β-adrenergic receptor promoted the phosphorylation of distinct subsets of potential c-Src substrates. This indicates that individual cell surface receptors and their distinct pathways could activate different pools of c-Src, whose access to different c-Src substrates varies. This would influence distinct subsets of various downstream targets of c-Src. Selective Ral-dependent activation of c-Src substrates may allow Src to be activated without Ras/MAPK activation. Like c-Src, Ral may be able to activate only a subset of potential downstream targets under distinct circumstances, or Ral activation by ligands may be necessary but not sufficient for c-Src activation (Goi *et al.*, 2000).

2.2.3 Cellular Distribution of Ral and c-Src

Ral and c-Src have similar cellular distributions (Goi *et al.*, 2000). The majority of c-Src and Ral is present on the cytoplasmic face of intracellular vesicles, with a small fraction present in the plasma membrane at any time (Feig and Emkey, 1993; Weernink

and Rijksen, 1995). Both proteins are enriched in endosomes and synaptic vesicles (Kaplan *et al.*, 1992; Lindstedt *et al.*, 1992; Bielinski *et al.*, 1993; Onofri *et al.,* 1997; Foster-Barber and Bishop, 1998). Elevated RalGEF activity (Goi *et al.*, 1999) and elevated activity of c-Src substrate, STAT3 (Ihara *et al.*, 1997), generate the same phenotype in PC12 cells as increased Ral activity: inhibition of nerve growth factor-induced neurite outgrowth (Goi *et al.*, 1999).

Ral proteins are widely distributed (Polakis *et al.*, 1989; Bhullar *et al.*, 1990; Bhullar, 1992; Olofsson *et al.*, 1988; Wildey *et al.*, 1993), and have diverse subcellular localizations, including plasma membrane (Polakis *et al.*, 1989; Feig and Emkey, 1993) and various cytoplasmic vesicles involved in endocytosis, exocytosis and secretion (Mark *et al.*, 1996; Polakis *et al.*, 1989; Feig and Emkey, 1993; Bielinski *et al.*, 1993: Volknandt *et al.*, 1993; Huber *et al.*, 1994). Src also associates with cellular membranes, in particular plasma membrane, perinuclear/Golgi membranes and endosomes (Willingham *et al.*, 1979; Courtneidge *et al.*, 1980; Resh and Erikson, 1985; David-Pfeuty and Nouvian-Dooghe, 1990; Kaplan *et al.*, 1992). In fact, 30-40% of cellular c-Src is localized to the perinuclear region (Tanaka and Kurth, 1984; Resh and Erikson, 1985). Src is primarily associated with membranes of the microtubule organizing centre that represents a late stage in the endocytic pathway, suggesting a role for c-Src in regulating the transport or function of specialized secretory vesicles (Kaplan *et al.*, 1992). The presence of c-Src in secretory organelles of chromaffin cells and platelets (Parsons and Creutz, 1986; Rendu *et al.*, 1989), and its association with endosomally-derived synaptic vesicles in differentiated PC-12 cells (Linstedt *et al.*, 1992), points to a role for Src in protein trafficking events (Bjorge *et al.*, 2000). Src interacts with ASAP1, an Arf

GTPase-activating protein, and the interaction of ASAP1 with Src, Arfs and PIP_2 is important in coordinating membrane trafficking with actin cytoskeletal remodelling (Brown *et al.*, 1998; Randazzo *et al.*, 2000). Src associates with and/or phosphorylates various proteins implicated in vesicle transport, including synapsin 1, dynamin, synaptophysin, synaptogyrin and cellugyrin (Barnekow *et al.*, 1990; Onofri *et al.*, 1997; Foster-Barber and Bishop, 1998; Janz and Sudhof, 1998). A potential target of Src is Golgi-67, a Golgi protein involved in vesicle docking/tethering (Jakymiw *et al.*, 2000), suggesting Src has a role in regulating membrane trafficking events, with possible links to the Golgi apparatus (David-Pfeuty and Nouvian-Dooghe, 1990; Abu-Amer *et al.*, 1997; Bjorge *et al.*, 2000).

2.2.4 Regulation of Ral by Protein Kinase C

Although in many cell types the three Ras effector pathways complement each other to promote cell proliferation (Wolthuis *et al.*, 1997), in PC12 pheochromocytoma cells the Ras/RalGEF signalling pathway antagonizes the Ras/Raf and Ras/PI3K pathways (Goi *et al.*, 1999). Raf kinase and PI3K mediate the action of nerve growth factor (NGF) by promoting neurite outgrowth and cell cycle arrest, whereas RalGEF antagonizes NGF action by promoting cellular proliferation and suppressing neurite outgrowth. This suggests that Ras activation of Raf and PI3K promotes the induction of differentiation of PC12 cells, whereas Ras activation of RalGDS-Ral may prevent differentiation (Reuther and Der, 2000). NGF induces a negative signal that preferentially suppresses the Ras/Ral-GEF signalling pathway, at least in part, via a PKC-dependent manner (Rusanescu *et al.*, 2001). The NGF- and EGF-induced activation of Ral in PC12

and COS-7 cells, respectively, is suppressed by phorbol 12, 13-diacetate (PDA)-stimulated PKC activity. In contrast, the Raf/ERK signalling pathway is activated in both cell types. In PC12 cells, inhibition of PKC by GF109203X enhanced basal Ral-GTP levels and increased the amount of time Ral remained active, but had little, if any, effect on NGF induction of ERK activity. Therefore, receptor activation of PKC preferentially suppresses Ras-induced Ral activation. PKC activity changed the ratio of Ras effector signalling in favour of ERK activation over Ral activation (Rusanescu *et al.*, 2001). Because PKC suppresses Ras activation of RalGDS without affecting binding between the two proteins or basal RalGDS activity, full RalGDS activation by Ras requires a secondary event that is blocked by PKC activity (Rusanescu *et al.*, 2001). However, regulation of RalGEF may differ in various cell types. Phorbol ester treatment activates Ral in some cells (Voss *et al.*, 1999) which must contain a phorbol-responsive RalGEF (Rusanescu *et al.*, 2001).

2.2.5 Connection Between Ral, EGFR, c-Src and STAT3 in Cancer

Ral, Src, EGFR and STAT3 may be intimately connected in the pathogenesis of various cancers. Constitutively activated Ral alleles by themselves are not considered oncogenes (Goi *et al.*, 2000), but cell proliferation was enhanced in 3Y1 cells also overexpressing EGFR (Lu *et al.*, 2000). The EGFR is frequently overexpressed in human breast and ovarian cancer (Reese and Slamon, 1997). Because Ral can activate c-Src, it is possible that the presence of a constitutively activated mutant allele of Ral or amplification of a RalGEF contributes to the deregulation of c-Src and STAT3 found in tumors (Goi *et al.*, 2000). That many breast cancers display elevated levels of EGFRs or

the related HER-2 receptors, and that expression of constitutively activated Ral enhances soft agar growth of cells overexpressing EGFRs (Lu *et al.*, 2000), suggests to Goi *et al.*, (2000) that Ral's presence could enhance the oncogenic potential of neoplastic cells containing amplified EGF or ERB2 genes (Garcia *et al.*, 1997; Biscardi *et al.*, 1998).

There are documented synergistic effects of c-Src and EGFR activities on the transformed phenotype of tissue culture cells (Roche *et al.*, 1995; Belsches *et al.*, 1997). Src-mediated phosphorylation of EGFR (Tyr^{845}) is involved in regulation of receptor function as well as in tumour progression (Biscardi *et al.*, 1999a). In C3H10T1/2 murine fibroblasts that contain kinase deficient c-Src and overexpressed EGFR, the kinase activity of c-Src is required for both the biological synergy with the receptor, and the phosphorylations on the receptor, but not for the association of c-Src with the receptor (Tice *et al.*, 1999). A Y845F EGFR mutant ablated, in a dominant negative fashion, the EGF-, serum- and lysophosphatidic acid-induced DNA synthesis. This suggests that c-Src-induced phosphorylation of Y^{845} is critical for the mitogenic response to both the EGFR and a GPCR (lysophosphatidic acid) (Tice *et al.*, 1999). Various human cancers, including carcinoma of the colon and mammary gland (Ottenhoff-Kalff *et al.*, 1992; Muthuswamy and Muller, 1994; Muthuswamy *et al.*, 1994; Watson and Miller, 1995; Garcia *et al.*, 1997; Biscardi *et al.*, 1999b) display elevated c-Src and STAT3 activity.

Activation of c-Src and EGFR is associated with progression of breast cancer. Both these events lead to activation of STAT proteins, which are increasingly being found to be activated in human cancers. Src activates STAT3-dependent transcription in mammary epithelial cells, and EGFR activation induces activation of STAT1 and 3 (Berclaz *et al.*, 2001). There is a highly significant correlation between nuclear STAT3

and EGFR expression and breast cancers compared to normal tissue (Berclaz *et al.*, 2001). This demonstrates a strong association between STAT3 activation and breast tumorigenesis, and suggests that STAT3 activation plays an important role in the tumorigenic conversion of breast tissue mediated by tyrosine kinase signalling pathways (Berclaz *et al.*, 2001). Src kinases also play an essential role in ErbB receptor signalling through the MAPK pathway. This involves the engagement of a novel, Shc-independent, Src-dependent route to MAPK (Olayioye *et al.*, 2001).

RalA was necessary for the activation of PLD in v-Src-transformed NIH3T3 cells (Jiang *et al.*, 1995a). As well, RalA, but not RalB, is activated by the interleukin-6 (IL6)/Janus kinase (JAK)/STAT3/RalGDS pathway, which is modulated by Ras in mouse M1 myeloid leukemia cells (Senga *et al.*, 2001). Thus STAT3 and Ral may regulate each other to exert biological functions (Senga *et al.*, 2001).

2.3 Ral-GTPase Interacts With and is Involved in the Signal Transduction Pathways of Na^+/K^+-ATPase

2.3.1 Cardiac Hypertrophy

2.3.1.1 Ral-GTPases and cardiac hypertrophy

Another growth related pathway in which Ral and RalGDS have been implicated is cardiac hypertrophy (Kawai *et al.*, 2003). Transfection of RalGDS and constitutively active RalGV23 mutant in cultured rat neonatal myocytes stimulated promoter activity of c-fos-, skACT-, and β-myosin heavy chain-luciferase (Kawai *et al.*, 2003). The Ral-GDS-induced or RalG23V-induced promoter activation was increased synergistically with activated RasG12V mutant. Dominant-negative RalS28N partially inhibited RasG12V-

induced promoter activity. Cardiac myocytes transfected with RalG23V showed increased cell size compared to non-transfected or vector-transfected cells. Cardiotrophin-1 (CT-1) upregulated RalGDS mRNA expression, an effect that was inhibited by a dominant-negative mutant of STAT3. CT-1 induced Ral activation, which was also elevated in hypertrophied hearts by mechanical stress in association with increased CT-1 expression and STAT3 phosphorylation in the rat aortic banding model. Therefore, STAT3 may play a key role in Ral-GDS expression and Ral activation (Kawai *et al.*, 2003).

2.3.1.2 Ouabain and other cardiac glycosides

Hypertrophy of the failing heart may not be only an adaptive and beneficial response of the diseased heart, but rather a part of the continuum of the derangements leading to advanced heart failure (Xie and Askari, 2002). Therapeutic concentrations of ouabain and other cardiac glycosides (e.g. digoxin) that cause partial inhibition of the sodium pump (Na^+/K^+-ATPase) and an increase in cardiac contractility, also stimulate cardiac myocyte hypertrophic growth and protein synthesis, induce a number of early-response proto-oncogenes, activate transcription factors activator protein-1 (AP-1) and NF-κB, and induce or repress the transcription of several late-response cardiac marker genes that are also regulated by other cardiac hypertrophic stimuli. Therefore, Na^+/K^+-ATPase regulates the growth and the phenotype of the cardiac myocyte (Xie and Askari, 2002). In adult and neonatal rat cardiac myocytes, glycosides regulate the transcription of genes that are markers for cardiac hypertrophy (*c*-fos, skACT, atrial natriuretic factor [ANF] and α3) (Peng *et al.*, 1996; Huang *et al.*, 1997a, 1997b; Kometiani *et al.*, 1998; Xie *et al.*, 1999; Tian *et al.*, 2001), and these hypertrophic gene regulatory actions

involve the activation of multiple interrelated signal transduction pathways, including the activation of Ras (Haas *et* al, 2000, 2002), PKC, calcineurin, and the three main branches of the MAPK signaling cascade (ERK, Jun amino-terminal kinase [JNK], p38)(De Windt *et al.*, 2000; Haas *et* al, 2000, 2002).

2.3.2 Structure and Function of Na^+/K^+-ATPase

We suspected that Ral may be involved in cardiac glycoside-stimulated hypertrophy, and results described in this thesis provide evidence for the first time that Ral-GTPases interact directly with the β1 subunit of Na^+/K^+-ATPase (β1). Na^+/K^+-ATPase (sodium pump or Na/K pump, EC 3.6.1.37) is an integral membrane protein that catalyses the coupled active transport of sodium (Na^+) and potassium (K^+) across the plasma membrane of most mammalian cells in an ATP-dependent manner (Skou, 1988; Lingrel and Kuntzweiler, 1994). It is responsible for establishing and maintaining the electrochemical gradient in animal cells, it contributes substantially to the maintenance of the membrane potential of the cell, provides the basis for neuronal communication, and contributes to the osmotic regulation of cell volume. As well, the electrochemical Na^+ gradient is the driving force behind secondary transport systems (Scheiner-Bobis, 2002).

Na^+/K^+-ATPase is composed of non-covalently-linked α, β and γ subunits. It is encoded by a multigene family with varying isoforms for the α (α1, α2, α3 and α4) and β (β1, β2 and β3) subunits (Sweadner, 1989; Lane *et al.*, 1989; Lingrel, 1992). Expression of these isoforms occurs in a tissue-specific manner (Munzer *et al.*, 1994). While the α1 and β1 isoforms are expressed in most tissues, α2 is predominant in skeletal muscle, cardiac muscle, adipose tissue and glial cells (Sweadner *et al.*, 1994; Peng *et al.*, 1997).

The $\alpha 3$ isoform is found in high concentrations in neurons of the central nervous system (Sweadner, 1995; Peng *et al.*, 1997) and cardiac muscle (Jewell *et al.*, 1992; Sweadner *et al.*, 1994). The $\alpha 4$ isoform is specific to the testis (Shamraj and Lingrel, 1994). The $\beta 2$ isoform functions as an adhesion molecule on glial cells specifically involved in mediating interactions between neurons and glia (Gloor *et al.*, 1990). The $\beta 3$ isoform is expressed predominantly in the testis but also in the brain, kidney, lung, spleen, liver and intestines (Lingrel. and Kuntzweiler, 1994; Malik *et al.*, 1997; Peng *et al.*, 1997; Arystarkhova and Sweadner, 1997).

Individual α- and β-subunits are synthesized in the endoplasmic reticulum (ER), where β-subunits are subject to two covalent modifications: disulfide bond formation and N-linked core glycosylation (Chow and Forte, 1995). Neither the α- nor the β-subunit has a cleavable signal sequence (Geering, 1990). The α- and β-subunits are assembled, possibly cotranslationally (Hiatt *et al.*, 1984), in the ER, from which the heterodimers move to the Golgi network for further post-assembly processing of the core oligosaccharides. In the Golgi, distinct paths are set up for trafficking of Na^+/K^+-ATPase in transport vesicles to the basolateral plasma membrane (Tamkun and Fambrough, 1986; Chow and Forte, 1993, 1995).

The α subunit (113 kDa) which mediates the catalytic activity, contains the ATP, cation and cardiac glycoside binding and phosphorylation sites. It spans the membrane ten times with both N- and C-termini on the cytoplasmic side (Kometiani *et al.*, 2000). The C-terminal half of the α-subunit specifies assembly with a particular β-subunit (Gottardi and Caplan, 1993). The β-subunit (35 kDa) is a glycoprotein (mass increased to about 55 kDa) that is essential for normal function and assembly of the pump (Kometiani

et al., 2000), but whose exact function is unknown (Horisberger *et al.*, 1991). The β1 isoform has three N-linked glycosylation sites that are all glycosylated (Tamkun and Fambrough, 1986; Miller and Farley, 1988). The sugar molecules are not essential for β1 function (Takeda *et al.*, 1988; Zamofing *et al.*, 1989; Noguchi *et al.*, 1987). The β-subunit spans the membrane once, with a short cytoplasmic N-terminal portion of about 33 amino acids, a transmembrane domain of approximately 30 amino acids and a large extracellular C-terminal region of about 240 amino acids (Hasler *et al.*, 2000), the latter containing three highly conserved disulfide loops (Ohta *et al.*, 1986; Kirley, 1989; Miller and Farley, 1990; Chow *et al.*, 1992) essential for enzymatic activity (Chow *et al.*, 1992; Kawamura and Nagano, 1984; Kawamura *et al.*, 1985; Kirley, 1990). An increase in strength of the disulfide bonds (tightening of the β-subunit conformation) in the β-subunit is correlated with increased stability of the holoenzyme (Chow and Forte, 1995). The β-subunit influences the apparent affinities for K^+ and Na^+ (Lutsenko and Caplan, 1993; Jaisser *et al.*, 1994; Eakle *et al.* 1994; Shainskaya and Karlish, 1996; Hasler *et al.*, 1998), such as being involved in K^+ occlusion (Chow *et al.*, 1992; Lutsenko and Caplan, 1993) and giving the enzyme a higher apparent K^+ affinity (Koenderink *et al.*, 1999). The disulfide bonds play a significant role in the folding of the enzyme (α- and β-subunits) within the membrane (Geering, 1991; Ueno *et al.*, 1997) and in modulation of enzyme activity (Koenderink *et al.*, 1999). Assembly of the α- and β-subunits is important for conformational stability of the functional holoenzyme (Ackermann and Geering, 1990; Tyagarajan *et al.*, 1995). The αβ complex is essential for enzyme activity (Noguchi *et al.*, 1987; Horowitz *et al.*, 1990; Klaassen *et al.*, 1993) and occurs before the subunits are transported from the ER to the plasma membrane (Tamkun and Fambrough, 1986;

Geering, 1991). In summary, β-subunits (and specifically β1) may be involved in maturation of the enzyme, localization of the enzyme to the membrane, protein folding, stabilization of the K^+-bound form of the enzyme, and modulation of enzyme activity (Geering, 1990, 1991; McDonough *et al.*, 1990; Lutsenko and Kaplan, 1993; Eakle *et al.*, 1994; Lingrel *et al.*, 1994; Koenderink *et al.*, 1999).

A 6.5-10 kDa γ-subunit is associated with the αβ complex and is involved in forming the site for cardiac glycoside binding (Mercer *et al.*, 1993). It is a type I transmembrane protein with its amino-terminus extracellular to the plasma membrane (Mobasheri *et al.,* 2000). It interacts only with assembled transport–competent αβ heterodimers (Beguin *et al.*, 1997). It is not an essential component for the functional or structural maturation of the Na^+/K^+-ATPase (Geering *et al.*, 1996; Ueno *et al.*, 1997), but may be involved in the activation of the enzyme by K^+ (Mobasheri *et al*, 2000). Because the γ-subunit is significantly identical to a family of single-pass, ion-transporting transmembrane proteins, it may also behave as a cation channel independent of the α- and β-subunits under certain physiological conditions (Mobasheri *et al.*, 2000). The γ-subunit does play a modulatory role in enzyme function. The αβγ complex has less affinity for Na^+ and K^+ than the αβ complex, suggesting selective expression of subunits in different tissues regulates Na^+/K^+-ATPase activity (Arystarkhova *et al.*, 1999). The γ-subunit thus has an important influence on enzyme activity and transepithelial ion transport (Mobasheri *et al.*, 2000).

2.3.3 Regulation of Expression of Na^+/K^+-ATPase Subunits

2.3.3.1 Hormones and ions

Expression of both α- and β-subunits are regulated by hormones and changes in intracellular ion concentrations (Orlowski and Lingrel, 1990; Pressley, 1992; Ismail-Biegi, 1992). Aldosterone and glucocorticoid hormones regulate the expression of the α- and β-subunits (Whorwood *et al.*, 1994; Whorwood and Stewart, 1995) in a tissue-specific manner, suggesting both mineralocorticoid and glucocorticoid receptors (MR and GR, respectively) are involved in the transcriptional regulation of the enzyme, and that non-receptor factors might be involved in conferring mineralocorticoid versus glucocorticoid specificity (Ikeda *et al.* 1991; Kolla and Litwack, 2000). There is a 21 base pair functional glucocorticoid and mineralocorticoid response element at position –650 of the β1 gene promoter (Derfoul *et al.*, 1998). Coexpression of both MRs and GRs activated by aldosterone and cortisone inhibited the significant transactivation of the β1 promoter that was seen with the independent expression of both receptors, indicating that interaction of MR with GR on the β1 promoter down-regulates transcription (Derfoul *et al.*, 1998).

2.3.3.2 Growth factors and cardiac glycosides

In cardiac myocytes, the cardiac glycoside, ouabain, and hypertrophic stimuli such as phorbol 12-myristate 13-acetate (PMA, which activates protein kinase C [PKC]), IGF-1 (which activates receptor tyrosine kinases), phenylephrine and endothelin-I (which use GPCRs) upregulate expression of the β1 subunit, down-regulate α3 subunit expression, and have no effect on the α1 subunit (Kometiani *et al.*, 2000). In a liver cell line, β1 was shown to be stimulated by different growth factors including serum and

PMA (Bhutada and Ismail-Beigi, 1991). Therefore, β1 is upregulated by growth factors and ouabain-activated growth-related pathways, suggesting that up-regulation of β1 may represent a common feature of cell growth (Kometiani *et al.*, 2000).

2.3.3.3 Ca^{2+}, CaM and PKC

Ouabain stimulates β1 expression through a transcriptional mechanism in cardiac myocytes, an effect that is dependent on PKC, CaM and a net influx of extracellular Ca^{2+} (Huang *et al.*, 1997a), and which is mediated through the ouabain-activated pathways of hypertrophic growth (Kometiani *et al.*, 2000). Immunohistochemical localization of Na^+/K^+-ATPase and CaM has been detected in the plasma membrane and plasmalemmal vesicles of rat vascular endothelium and cardiac muscle cells (Nakagawa *et al.*, 1990). Thus, CaM plays an essential role in regulation of β1, in addition to other cardiac genes, such as α3 subunit of Na^+/K^+-ATPase (α3) and skeletal alpha actin (skACT) (Huang *et al.*, 1997b; Kometiani *et al.*, 1998). PKC plays a key role in the regulation of both basal and stimulated expression of β1. PKC is probably activated as a down-stream event from ouabain-induced increase in Ca^{2+} and activation of CaM (Kometiani *et al.*, 2000).

2.3.3.4 Ras/Raf/MEK/ERK pathway

A dominant negative Ras and a MAPK kinase (MEK) inhibitor (PD 98059) only partially blocked ouabain-stimulated β1 expression, suggesting β1 expression is regulated by both Ras/MAPK-dependent and -independent events, and that a Ras-independent pathway in β1 regulation may involve Ca^{2+} and CaM (Kometiani *et al.*, 2000). Inhibition of Na^+/K^+-ATPase by lowering extracellular K^+ also increases β1 expression in several different cells including neonatal rat cardiac myocytes (Qin *et al.*, 1994; Pressley *et al.*, 1988; Xie *et al.*, 2000). That both Ras/MAPK-dependent and Ras/MAPK–independent

mechanisms are involved in regulation of β1 (Kometiani *et al.*, 2000), whereas α3 is regulated only by Ras-dependent pathways (Huang *et al.*, 1997b; Kometiani *et al.*, 1998), suggests that regulation of cardiac genes occurs by gene-specific signals (Kometiani *et al.*, 2000).

2.3.4 Na^+/K^+-ATPase as an Ion Pump

2.3.4.1 The catalytic mechanism of Na^+/K^+-ATPase

Na^+/K^+-ATPase has two conformation states, E_1 and E_2 (Scheiner-Bobis, 2002) (Fig. 5). Na^+ and ATP bind with very high affinity to the E_1 conformation of the enzyme (Fig. 5, step 1), during which phosphorylation at an aspartate residue occurs via the transfer of the γ-phosphate of ATP (Fig. 5, step 2). Magnesium is very important for this reaction. Therefore, three Na^+ ions are occluded while the enzyme remains in a phosphorylated condition. After the E_2-P3Na^+ conformation is attained, the enzyme loses its affinity for Na^+ and the affinity for K^+ is increased. Thus, three Na^+ ions are released to the extracellular medium (Fig. 5, step 3) and two K^+ ions are taken up (Fig. 5, step 4). The binding of K^+ to the enzyme induces a spontaneous dephosphorylation of the E_2-P conformation. The dephosphorylation of E_2-P leads to the occlusion of two K^+ ions, leading to $E_2(2K^+)$ (Fig. 5, step 5). Intracellular ATP increases the extent of the release of K^+ from the $E_2(2K^+)$ conformation (Fig. 5, step 6) and thereby, also the return of the $E_2(2K^+)$ conformation to the E_1ATPNa^+ conformation. The affinity of the $E_2(2K^+)$ conformation for K^+ is very low. Through the juxtapositioning of these three reaction sequences, the full catalytic cycle of Na^+/K^+-ATPase is obtained (Fig. 5)

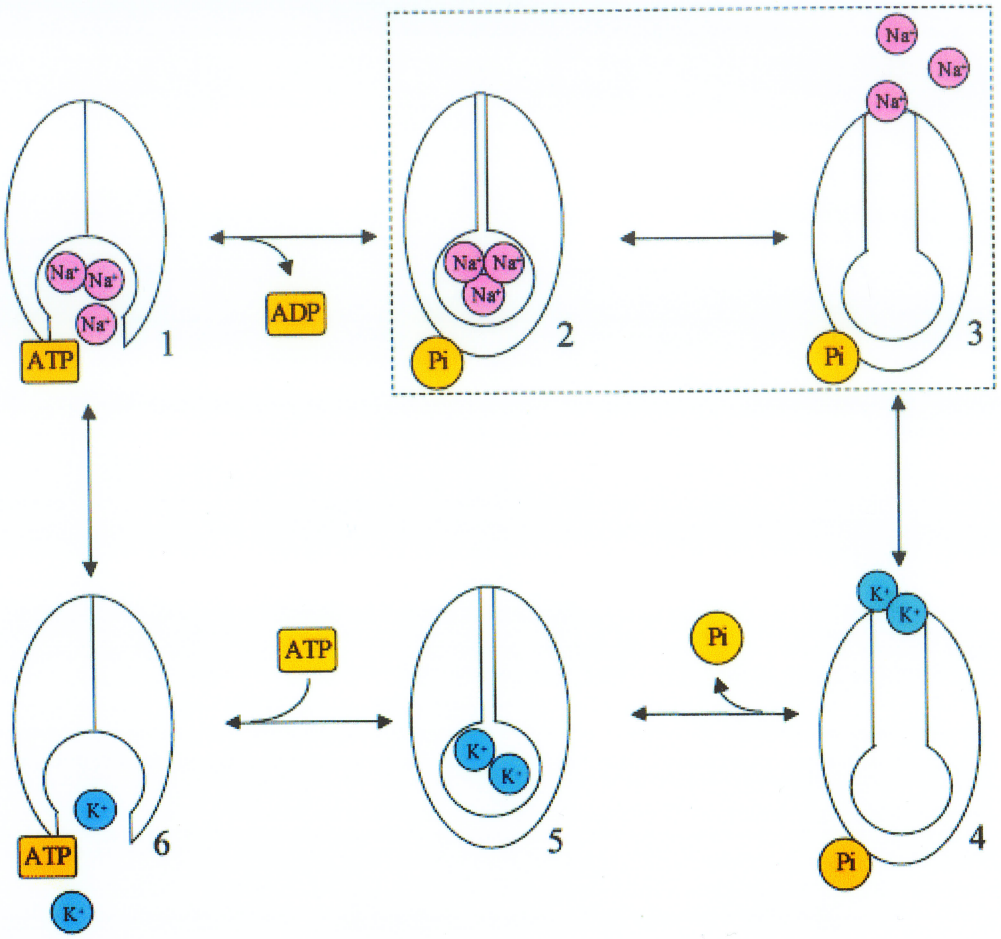


Fig. 5. Na^+/K^+-ATPase cycle. Na^+/K^+-ATPase binds Na^+ and ATP in the E_1 conformational state (step 1) and is phosphorylated at an aspartate residue by the γ-phosphate of ATP. This leads to the occlusion of three Na^+ ions (step 2) and then to the release to the extracellular side (step 3). This new conformational state (E_2-P) binds K^+ with high affinity (step 4). Binding of K^+ leads to dephosphorylation of the enzyme and to occlusion of two K^+ cations (step 5). K^+ is then released to the cytosol after ATP binds to the enzyme with low affinity (step 6). The dashed box highlights the electrogenic steps of the catalytic cycle (Scheiner-Bobis, 2002)

2.3.4.2 Cardiac glycosides and inotropy

In the heart, Na^+/K^+-ATPase serves as a receptor for the positive inotropic effects of cardiac glycosides (Braunwald, 1985; Schwartz *et al.*, 1988; Kelly and Smith, 1993; The Digitalis Investigation Group, 1997). Ouabain and related cardiac glycosides (e.g. digitalis) inhibit Na^+/K^+-ATPase by reversibly binding to the extracellular domain of the enzyme, which inhibits ATP hydrolysis and thus ion transport (Peng *et al.*, 1996; Huang *et al.*, 1997a, 1997b; Kometiani *et al.*, 1998; Xie *et al.*, 1999; Scheiner-Bobis, 2002). The partial inhibition of the myocardial enzyme by glycosides causes a small increase in intracellular Na^+ which in turn affects the sarcolemmal and plasma membrane Na^+/Ca^{2+}-exchangers, leading to a significant increase in intracellular Ca^{2+} and in the force of contraction (Skou, 1988; Lingrel and Kuntzweiler, 1994; Kometiani *et al.*, 2000).

2.3.5 Ion Pump and Signal Transducer

2.3.5.1 Pathways that are independent of changes in intracellular ion concentrations

Although an increase in Ca^{2+} is necessary but not sufficient for the ouabain-induced hypertrophy and the associated gene regulation, large segments of the early events that result from ouabain interaction with cardiac Na^+/K^+-ATPase are independent of any changes in intracellular Ca^{2+}, Na^+ and K^+ concentrations, but depend on the enzyme's interaction with other proteins (Haas *et al.*, 2000; Liu *et al.*, 2000). Increases in protein tyrosine phosphorylation of a number of proteins, an early event induced by ouabain, activation of Ras and the resulting Ras-dependent generation of reactive oxygen species (ROS) by mitochondria, do not require ouabain-induced increases in intracellular Ca^{2+} or Na^+ concentrations in neonatal cardiac myocytes (Liu *et al.*, 2000). This indicates

that ouabain can initiate at least some signal transduction pathways independent of changes in intracellular ion concentrations (Liu *et al.*, 2000; Tian *et al.*, 2001). These findings lead to two important conclusions. First, that the primary signaling events emanating from ouabain-induced partial inhibition of Na^+/K^+-ATPase are altered protein-protein interactions; i.e. changes in the interactions of the Na^+/K^+-ATPase subunits with neighboring proteins at or within the plasma membrane (Haas *et al.*, 2000). Src activation may be due to a change in the interaction of an intermediate protein(s) between Na^+/K^+-ATPase and Src (Haas *et al.*, 2000). Second, that Na^+/K^+-ATPase has two distinct roles in the plasma membrane: one being its classical function as an ion pump (Skou and Esmann, 1992; Lingrel and Kuntzweiler, 1994), and the other as a signal transducer (Liu *et al.*, 2000).

2.3.5.2 Signal Trasnsducer

Na^+/K^+-ATPase responds to extracellular stimuli such as ouabain and low extracellular K^+ to relay messages, through protein-protein interactions and second messengers, to intracellular signaling complexes, the mitochondria, and the nucleus (Peng *et al.*, 1996; Huang *et al.* 1997a, 1997b; Kometiani *et al.*, 1998; Xie *et al.*, 1999; Haas *et al.*, 2000; Liu *et al.* 2000). The earliest specific ouabain-induced event is the activation of Src kinase, which leads to tyrosine phosphorylation of a number of cellular proteins, including the EGFR (Haas *et al.*, 2000) (Fig. 6). Therefore, c-Src and the EGFR play key roles in the ouabain-activated signal transducing function of Na^+/K^+-ATPase (Haas *et al.*, 2000, 2002). In cardiac myocytes and A7r5 cells, partial inhibition of Na^+/K^+-ATPase by ouabain stimulated Src kinase activity, Src translocation to membranous fractions, Src association with the EGFR, and the tyrosine phosphorylation

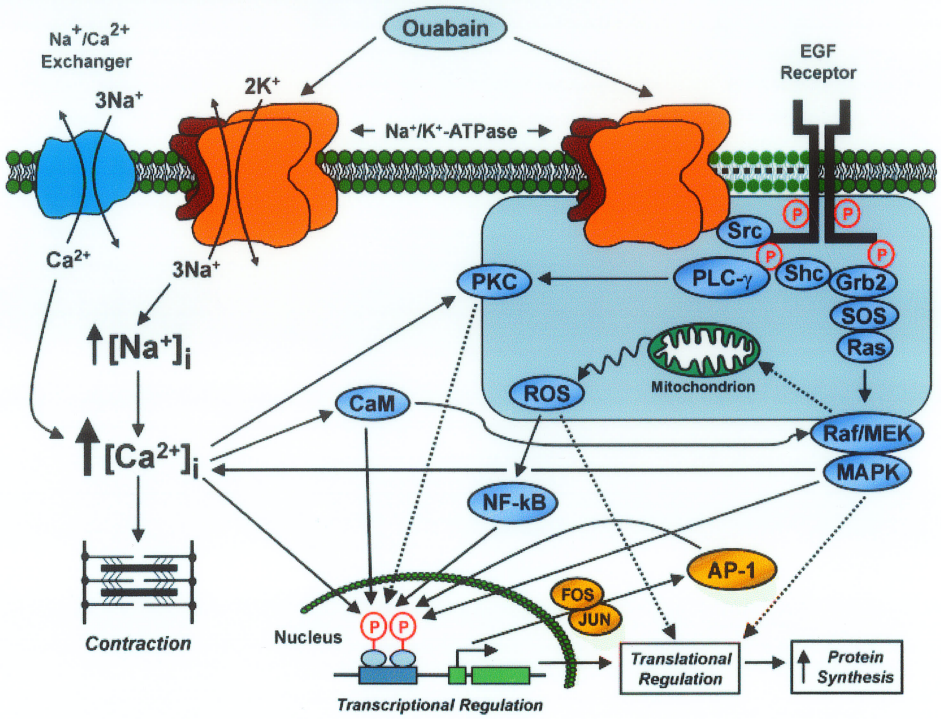


Fig. 6. The signal transducing function of Na^+/K^+-ATPase and its consequences in cardiac myocytes. Two pools of the enzyme, one pumping ions and the other interacting with neighboring proteins. The partial inhibition of the pump by ouabain causes a modest change, if any, in $[Na^+]_i$ and $[K^+]_i$, but a significant change in $[Ca^{2+}]_i$ due to the presence of the Na^+/Ca^{2+}-exchanger. Ouabain interaction with the other pool alters protein-protein interactions to activate the indicated signaling pathways. The events placed in the grey box have been shown to be independent of changes in $[Na^+]_i$, $[K^+]_i$, and $[Ca^{2+}]_i$ that may occur. These activated pathways, the resulting increase in ROS, and the concomitant increase in $[Ca^{2+}]_i$ lead to activations of NF-κB and AP-1, transcriptional regulation of early response genes (c-fos, c-jun), and cardiac growth-related genes (those of ANF, skACT, and α_3), stimulation of protein synthesis, and myocyte hypertrophy. The solid arrows indicate experimentally supported events induced by ouabain in myocytes, and the broken arrows indicate those with limited or indirect support. (Xie & Askari, 2002).

of this receptor on sites (Tyr^{845} & Tyr^{1101}) other than its major autophosphorylation site (Tyr^{1173}) that is induced upon binding of its cognate receptor (Biscardi *et al.*, 1999a; Haas *et al.*, 2000). This version of transactivated EGFR provides the scaffolding for the recruitment of adaptor proteins (Shc, Grb2), son of sevenless (SOS) and Ras to the plasma membrane (Kometiani *et al.*, 1998; Schlaepfer *et al.*, 1998; Eguchi *et al.*, 1998; Tice *et al.*, 1999; Haas *et al.*, 2002), which may be a common feature involved in the function of Na^+/K^+-ATPase (Haas *et al.*, 2000).

Because oubain stimulated Src phosphorylation at Tyr^{418} but not Tyr^{129}, it is proposed that ouabain-induced Src activation is due to the increased interaction between Src and Na^+/K^+-ATPase (Haas *et al.*, 2002). Immunoprecipitation experiments in LLC-PK1 cells showed that ouabain stimulated the binding of Src to the α1 subunit of Na^+/K^+-ATPase in a dose and time-dependent manner (Haas *et al.*, 2002).

2.3.5.3 Reactive oxygen species

Ras activation leads to generation of mitochondrial ROS, which is downstream to increases in protein tyrosine kinase (PTK) phosphorylation and the associated Src activation (Xie *et al.*, 1999; Liu *et al.*, 2000). Therefore, signal pathways initiated at Na^+/K^+-ATPase through protein-protein interactions extend to the mitochondria to generate ROS (Liu *et* al. 2000). That large segments of the proximal signaling events emanating from Na^+/K^+-ATPase do not require a rise in intracellular Ca^{2+} concentration indicate that this rise is not an early and all-important second messenger for the gene regulatory role of the cardiac Na^+/K^+-ATPase, but that it cooperates with the increased ROS to regulate downstream events and cross-talk among the pathways (Liu *et al.*, 2000). It also appears that the ouabain-induced pathways leading to increased protein tyrosine

phosphorylation and ROS production are parallel to those leading to a prominent rise in Ca^{2+} concentration through the altered function of the Na^{+}/Ca^{2+}-exchanger (Akera and Brody, 1976; Akera and Ng, 1991; Eisner and Smith, 1992; Liu *et al.*, 2000).

Although ROS have normal roles as second messengers within several signal pathways involved in the control of gene transcription (Sen and Packer, 1996; Lander, 1997), they are essential second messengers in some but not all signal pathways activated by ouabain-inhibited Na^{+}/K^{+}-ATPase (Xie *et al.*, 1999). ROS are not involved in the effects of ouabain on $[Ca^{2+}]i$ (Tian *et al.*, 2001), but they play an essential role in ouabain-mediated regulation of cardiac genes and cell growth (Xie *et al.*, 1999). In cardiac myocytes, ROS are required for ouabain-induced transcriptional regulation of late-response marker genes, induction of genes skACT and ANF and repression of $\alpha 3$, and activation of MAPKs, transcription factor NF-κB and Ras-dependent protein synthesis. ROS are not involved in induction of *c-fos* or activation of AP-1, or in the inhibition of Na^{+}/K^{+}-ATPase and the resulting increase in intracellular Ca^{2+}. Increased ROS generation is not a common response of the myocyte to all hypertrophic stimuli, as PMA did not increase ROS production (Xie *et al.*, 1999). The pathway beginning with Ras and leading to NF-κB activation is essential to ouabain-induced hypertrophy (Xie and Askari, 2002). That antioxidants attenuate but do not abolish ouabain-induced ERK activation (Xie *et al.*, 1999), suggests that there is a signal amplification cycle consisting of Ras-dependent ROS generation and ROS-dependent activation of Ras (Xie and Askari, 2002).

2.3.5.4 Activation of Raf/MEK/ERK cascade and the role of increased Ca^{2+}

Ouabain caused a 3.5 fold increase in Ras binding to Raf in A7r5 cells, resulting in activation of the Ras/Raf/MEK/ERK pathway (Haas *et al.*, 2002). Inhibition of PTKs by PP2 (a specific Src kinase inhibitor), genestein (a non-specific tyrosine kinase inhibitor) or herbimycin A (a specific Src family kinase inhibitor) abolished ouabain-induced stimulation of p42/44 MAPKs (Haas *et al.*, 2000, 2002). Therefore, ouabain-induced activation of Ras, through transactivation of the EGFR by c-Src, leads to activation of two Ras-dependent branched pathways: one communicating with the mitochondria to induce generation of mitochondrial ROS, and the other being the Raf/MEK/ERK pathway (Eisner and Smith, 1992; Peng *et al.*, 1996; Huang *et al.* 1997a, 1997b; Kometiani *et al.*, 1998; Xie *et al.*, 1999; Liu *et al.* 2000; Haas *et al.*, 2000, 2002) (Fig. 6). Activation of the Raf/MEK/ERK cascade, in contrast to the generation of ROS, requires the rise in $[Ca^{2+}]_i$ that is caused by ouabain's inhibition of the ion transporting function of the Na^+/K^+-ATPase (Kometiani *et al.*, 1998; Xie and Askari, 2002).

Through use of chemical inhibitors of EGFR and platelet-derived growth factor (PDGFR), Haas *et al.* (2002) determined that the ouabain-induced activation of MAPKs is specific for EGFR. These findings demonstrate Na^+/K^+-ATPase is linked to the Ras/MAPK cascade through Src and the EGFR (Haas *et al.*, 2000), and that the Src-mediated inter-receptor cross-talk between Na^+/K^+-ATPase and EGFR is essential for the ouabain-induced activation of MAPKs, resulting in activation of multiple downstream signaling processes (Haas *et al.*, 2002). Therefore, activation of c-Src is the initial critical step that relays the signal emanating from the interaction of ouabain with Na^+/K^+-ATPase

to the EGFR and the subsequent activation of the Ras/MAPK pathway (Haas *et al.*, 2002).

2.3.5.5 Protein Kinase C

Ouabain-induced activation of PKC is also required for ERK activation (Mohammadi *et al.*, 2001), most likely through PKC activation of Raf recruited to the plasma membrane by Ras (Xie and Askari, 2002). Ouabain causes rapid and sustained stimulation and translocation of several PKC isoforms (α, δ and ϵ) from cytosolic to particulate fractions in rat neonatal cardiac myocytes (Mohammadi *et al.*, 2001). This activation of PKC precedes the activation of ERK1/2, and is dependent on the activation of PLC and stimulation of phosphoinositide turnover. That Ras is also essential for ouabain-induced activation of ERK1/2 suggests Ras and PKC must cooperate in parallel pathways to activate Raf. The ouabain-induced PKC stimulation is Ca^{2+}-dependent, as is therefore the activation of ERK1/2 (Mohammadi *et al.*, 2001). There is cross-talk between the ion pumping functions and the signal-transducing functions of Na^+/K^+-ATPase that may depend on a Ca^{2+} effect; e.g. the ouabain-induced rise in intracellular Ca^{2+} (ion pump) is needed for the PLC-induced activation of PKC, which then activates, like Ras, the Raf/MEK/ERK1/2 signal transduction pathways (Mohammadi *et al.*, 2001). PKC and ERK1/2 may then help suppress the rise in Ca^{2+}, and PKC may inhibit Na^+/K^+-ATPase function in negative feedback loops. It is known Na^+/K^+-ATPase is a substrate for PKC (Ewart and Klip, 1995; Therien and Blostein, 2000), and PKC induces changes in the activity or endocytosis of Na^+/K^+-ATPase (Lundmark *et al.*, 1995; Efendiev *et al.*, 2000). The Ca^{2+}-CaM kinase also seems to be involved in ouabain-induced activation of

ERK and regulation of early and late response genes (Huang *et al.*, 1997a, 1997b; Kometiani *et al.*, 1998).

Regulation of the transcription of growth related genes and the induction of myocyte hypertrophy is dependent on the downstream collaboration of ouabain-induced mitochondrial ROS production and increased intracellular Ca^{2+} levels (Liu *et al.*, 2000) (Fig. 6). Ouabain causes increased levels of proto-oncogenes *c-fos* and *c-jun* mRNAs and the transcription factor AP-1 in a Ca^{2+}- and PKC-dependent manner (Peng *et al.*, 1996). All ouabain's gene regulatory effects (regulation of *c*-fos, skACT, ANF and α3 subunit genes), and the ouabain-induced activation of Raf and MAPK in rat neonatal cardiac myocytes are totally dependent on extracellular Ca^{2+}, CaM and PKC (Peng *et al.*, 1996; Huang *et al.*, 1997a, 1997b; Kometiani *et al.*, 1998).

The signal-transducing function of Na^+/K^+-ATPase also contributes to the classic ouabain-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in cardiac myocytes (Tian *et al.*, 2001). In adult rat cardiac myocytes, ouabain caused stimulation of protein tyrosine phosphorylation and activation of p42/44 MAPKs, increased intracellular ROS concentrations, and increased systolic and diastolic $[Ca^{2+}]i$ in a time- and dose-dependent manner (Tian *et al.*, 2001). The effects of ouabain on $[Ca^{2+}]i$ are mediated by ouabain-activated growth pathways, requiring stimulation of protein tyrosine kinases, Ras and the ERK pathway. Inhibition of either protein tyrosine phosphorylation or MEK and ERK diminishes ouabain-induced increases in $[Ca^{2+}]_i$ (Tian *et al.*, 2001). For example, either genistein or herbimycin A diminished ouabain-induced increases in $[Ca^{2+}]i$, while PP2 abolished the effects of ouabain on both p42/44 MAPKs and $[Ca^{2+}]i$. Activated Ras is essential for the effects of ouabain on $[Ca^{2+}]i$, because a dominant-negative RasN17

abolished the ouabain-induced rise in $[Ca^{2+}]i$. Ca^{2+} channels may be activated by MAPKs in response to ouabain, thus amplifying the effects of ouabain on $[Ca^{2+}]_i$ (Tian *et al.*, 2001). It is believed that both the inhibition of the ion pumping function and activation of the signal transduction functions of the enzyme are required for ouabain regulation of $[Ca^{2+}]i$ in cardiac myocytes (Tian *et al.*, 2001). Although inhibition of the enzyme by ouabain can cause some increase in $[Ca^{2+}]i$ by inhibition of Na^+/Ca^{2+} exchanger-mediated Ca^{2+} extrusion due to a small rise in intracellular Na^+ (Lee, 1985; Satoh, 2000), activation of MAPKs by ouabain may amplify ouabain's effects on Ca^{2+} levels by altering the function of membrane transporters or ion channels (Tian *et al.*, 2001). There is cross-talk between Ca^{2+} and MAPKs, because increases in $[Ca^{2+}]i$ can further activate MAPKs (Bogoyevitch *et al.*, 1996; Kometiani *et al.*, 1998; Sadoshima *et al.*, 1995). Therefore, increase in Ca^{2+} levels and activation of MAPKs may form a signal amplification loop or another positive feed-back cycle in cardiac myocytes; i.e. the requirement of a rise in $[Ca^{2+}]_i$ for ERK activation, and the necessity of ERK activation for rise in $[Ca^{2+}]_i$ (Tian *et al.*, 2001; Xie and Askari, 2002).

2.3.6 Two Pools of Na^+/K^+-ATPase with Two Distinct but Coupled Functions

The interaction of non-toxic concentrations of ouabain with the cardiac myocyte Na^+/K^+-ATPase leads to the generation of two intracellular second messengers, increased ROS and increased $[Ca^{2+}]_i$, both of which are essential for the full expression of hypertrophic and gene regulatory actions of ouabain; and each is generated in parallel with the other (Liu *et al.*, 2000). Therefore, there are two pools of Na^+/K^+-ATPase within the plasma membrane with two distinct functions: one being the classical pool of the

enzyme as an energy transducing ion pump whose partial inhibition by ouabain initiates the increase in $[Ca^{2+}]_i$, and the other the signal transducing pool of the enzyme which, through protein-protein interactions, leads to activation of a host of signaling intermediates and a rise in intracellular ROS (Fig. 6). In cardiac myocytes, the functions of these two pools are tightly coupled through feed-back cycles to regulate cardiac contractility and growth (Xie and Askari, 2002).

2.3.7 Signal Transducing Role of Na^+/K^+-ATPase in Cells Other Than Cardiac Myocytes

Linkage to signaling intermediates and pathways through protein-protein interactions is a common property of Na^+/K^+-ATPase in most, if not all cells. Ouabain-induced activation of the Ras/ERK pathway and generation of ROS has been demonstrated in A7r5 and HeLa cells (Haas *et al.*, 2000; Liu *et al.*, 2000). In non-myocytes, ouabain activates a number of protein kinase signaling pathways (Kuroki *et al.*, 1997; Li and Wattenberg, 1998; Contreras *et al.*, 1999), suggesting that changes in intracellular concentrations of Na^+, K^+, or both may also modulate the signal transducing function of Na^+/K^+-ATPase that is initiated by protein-protein interactions (Xie and Askari, 2002).

2.3.8 The Physiological Stimuli for Signal Transduction by Na^+/K^+-ATPase

Being endogenous, steroid hormones (Schoner, 2002), ouabain and related digitalis compounds bind to the extracellular domain of Na^+/K^+-ATPase with exquisite specificity (Xie and Askari, 2002). The other highly selective ligand for the extracellular

domain of Na^+/K^+-ATPase is K^+. Lowering of extracellular $[K^+]$ has been shown to act in a manner similar to ouabain and activate the proximal segments of the signaling pathways in cardiac myocytes (Haas *et al.*, 2000). Like ouabain, low K^+ stimulated protein tyrosine phosphorylation and MAP kinases, but unlike ouabain, failed to stimulate protein synthesis in cardiac myocytes (Xie *et al.*, 2000; Haas *et al.*, 2000). This suggests ouabain and low K^+ share some pathways, but also activate different, stimulus-specific pathways in cardiac myocytes (Kometiani *et al.*, 2000). In smooth muscle and epithelial cells, lowering of $[K^+]$ does not mimic the signaling effects of ouabain (Aydemir-Koksoy *et al.*, 2001; Aizman *et al.*, 2001), emphasizing the diversity of the signal transducing functions of Na^+/K^+-ATPase (Xie and Askari, 2002). As well, increases in intracellular Ca^{2+} caused by ouabain and by other means have different effects on p42/44 MAPKs, suggesting different intracellular pools of Ca^{2+} have different effects within the signal pathways (Kometiani *et al.*, 1998).

2.4 Conclusion and Hypothesis

We hypothesize that like RalA (Wang *et al.*, 1997) RalB is also a CaM binding protein, and that both require CaM for activation and function. Because EGF activates c-Src via the Ras/RalGEF/Ral pathway which leads to activation of STAT3 and gene transcription (Goi *et al.*, 2000), and because Ral is required for Ras-mediated cell proliferation and oncogenesis (Song *et al.*, 1994; Urano *et al.*, 1996; White *et al.*, 1996; Feig *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997; Aguirre Ghiso *et al.*, 1999; Reuther and Der, 2000), we propose that CaM also regulates Ral activation in this important pathway. The dependence on CaM may be cell type- and agonist-specific, and to that end we wish

to examine the effects of other proteins on this pathway in different cell types. From results obtained in searching for novel Ral binding proteins in order to discover new Ral functions, we also speculate that both RalA and RalB bind the β1 subunit of Na^+/K^+-ATPase, and are involved in the expression and activity of β1 and, therefore, the Na/K pump. Because ouabain-induced inhibition of Na^+/K^+-ATPase activates cardiac hypertrophic genes (Kometiani *et al.*, 2000; Haas *et al.*, 2000, 2002; Xie and Askari, 2002), and Ral and RalGDS are involved in cardiac hypertrophy (Kawai *et al.*, 2003), we suspect that Ral and CaM are involved in the cardiac glycoside-mediated, Na^+/K^+-ATPase-induced signal transduction and hypertrophic pathways in various cell types via the Na^+/K^+-ATPase/c-Src/EGFR/Ras pathway.

Therefore we wish to examine our hypotheses that Ral interacts with CaM and β1 in human platelets and cells in tissue culture, and that Ral and CaM are required in various signal transduction pathways.

3. RESEARCH OBJECTIVES AND EXPERIMENTAL APPROACH

3.1 Research Objectives

My research goal is to investigate factors regulating activation of RalA and RalB in various signal transduction pathways in human platelets and mammalian tissue culture cells.

The working hypothesis is that (i) both Ral-GTPases bind CaM in a Ca^{2+}-dependent manner, and that their activation by thrombin in platelets and EGF in tissue culture cells is dependent on CaM and other signalling proteins, depending on cell type

and environment; (ii) both RalA and RalB interact with β1, and that they and CaM are involved in the signal transducing functions of the Na/K pump.

3.1.1 Specific Objectives

A. Examine CaM interaction with RalA and RalB, and the requirement of CaM for Ral activation in human platelets.

1. Show RalA and RalB bind CaM *in vivo* and *in vitro*.
2. Determine if Ral/CaM interactions are Ca^{2+}-dependent.
3. Determine CaM binding regions on Ral to see if there is more than one CaM BD in RalA and RalB.
4. Determine the Ca^{2+}-dependence of additional CaM BDs.
5. Show that CaM is required for Ral function; specifically that the thrombin-induced activation of Ral in human platelets dependes on CaM.

B. Examine the regulation of EGF-induced activation of Ral in mammalian tissue culture cells.

1. Determine what factors regulate EGF-induced Ral activation in A7r5, MCF7 and HeLa cells (e.g. Ca^{2+}, CaM, PLC, PKC, Ral-GEFs).
2. Determine if Ral activation is dependent on cell type and environment.
3. Determine if Ca^{2+} and CaM are required for EGF-induced c-Src activation.

C. Examine the Role of Ral and CaM in the signal transducing functions of Na^+/K^+-ATPase in A7r5 and HeLa cells.

1. Search for novel Ral binding proteins.
2. Examine interaction between Ral and β1.

3. Study effects of ouabain-induced inhibition of Na^+/K^+-ATPase on Ral activation and expression, as well as on expression of Ras and CaM.

4. Examine effects of Ral and CaM on ouabain-induced activation of c-Src.

3.2 Experimental Approach

3.2.1 Ral Interacts Directly with Calmodulin, and Requires Calmodulin for Activation

1. By use of restriction endonuclease techniques, construct truncated forms of Ral cDNAs, and prepare these and full-length Ral and CaM cDNAs for subcloning into expression plasmids.

2. Insert CaM cDNA and full-length and truncated Ral cDNAs into pGADT7 and pGBKT7 expression plasmids, respectively.

3. Demonstrate expression of haemagglutin (HA)-CaM and c-Myc-Ral fusion proteins (both full-length and truncated versions) occur from the expression plasmids by *in vitro* transcription/translation.

4. Demonstrate RalA and RalB interact with CaM *in vitro* in a Ca^{2+}-dependent manner by an *in vitro* binding assay.

5. Demonstrate Ral and CaM form a complex in human platelets by co-immunoprecipitation assay.

6. Demonstrate that GAL4 AD[CaM] and BD[Ral] fusion proteins are expressed in transformed yeast cells by western blotting.

7. Demonstrate RalA and RalB directly interact with CaM *in vivo* by a Y2H assay.

8. Determine if removal of C-terminal CaM BD of Ral inhibits Ral/CaM interactions and to demonstrate Ral has more than one CaM BD.
9. Determine the Ca^{2+}-dependence of N-terminal and C-terminal CaM BDs.
10. Demonstrate our GST-RRBD construct pulls down activated but not inactive Ral proteins.
11. Demonstrate CaM is required for the thrombin-induced activation of Ral in human platelets.

3.2.2 Regulation of EGF-induced Ral Activation in Mammalian Tissue Culture Cells

1. Using specific inhibitors, determine if (a) CaM, (b) Ca^{2+}, (c) PLC and (d) PKC are required for the EGF-induced activation of RalA and RalB in A7r5, MCF7 and HeLa cells.
2. Using PMA, determine if direct activation of PKC activates RalA and RalB in a time- and Ca^{2+}/CaM-dependent manner.
3. Via dominant-negative Ral transfection, determine if Ras-dependent RalGEFs are required for the EGF-induced activation of RalA in A7r5 and HeLa cells.
4. Via $p130^{Cas}$ transfection, determine if Ras-independent RalGEF, BCAR3/AND-34 is required for the EGF-induced activation of RalA and RalB in A7r5, MCF7 and HeLa cells.
5. Verification by western blotting that transfected cDNAs are abundantly translated and expressed in A7r5, HeLa and MCF7 cells.
6. Determine by use of phosphor-specific anti-c-Src antibody the Ca^{2+}/CaM-dependence of EGF-induced c-Src activation in A7r5 and HeLa cells.

3.2.3 Ral Interacts Directly with the β1 subunit of Na^+/K^+-ATPase, and is Involved in the Signal Transduction Pathway of the Na/K Pump

1. By screening a human testis cDNA expression library via yeast two-hybrid (Y2H) assay, search for novel Ral binding proteins.
2. Demonstrate full-length RalA and RalB interact directly with β1 *in vivo* via Y2H assay.
3. Demonstrate by western blotting thesubcellular distribution of Na^+/K^+-ATPase in human platelets.
4. Verify by western blotting and Coomassie staining that GST-β1 fusion proteins are expressed in *E. coli* strains used.
5. Demonstrate that β1 interacts with RalA and RalB *in vitro*, using GST- and Sepharose-coupled fusion protein pull-down assays in (a) human platelets, and (b) canine cardiac sarcoplasmic reticulum (CSR).
6. Demonstrate recombinant *in vitro* transcribed/translated [^{35}S]Met-labelled β1 interacts with GST-RalA and GST-RalB via an *in vitro* binding assay.
7. Determine whether Ral co-immunoprecipitates with (a) β1 and (b) c-Src in HeLa cells.
8. Determine effect of ouabain-induced inhibition of Na^+/K^+-ATPase on activation state of Ral in A7r5 and HeLa cells, using GST-RRBD pull-down assays.
9. Determine if increased intracellular Ca^{2+} causes Ral activation in HeLa cells treated with ouabain.

10. Determine what effect (a) short-term (15 minutes) and (b) long-term (24 hours) ouabain treatment has on EGF-induced Ral activation in A7r5 and HeLa cells.

11. Use western blotting to determine what effect long-term (18 hours) ouabain treatment has on total protein expression of (a) Ral, (b) β1, (c) Ras, and (d) CaM in A7r5 and HeLa cells.

12. Use western bloting to determine if Ral affects the expression of β1 and Ras in A7r5 and HeLa cells treated with long-term ouabain.

13. Using phospho-specific c-Src antibody, determine if CaM is required for the ouabain-induced activation of c-Src.

14. Using dominant-negative Ral transfection and phospho-specific c-Src antibody, determine the effect of Ral on ouabain-induced activation of c-Src.

4. MATERIALS AND METHODS

4.1 Materials

Dulbecco's modified eagle's medium (DMEM), minimum essential medium with Earle's salts (MEM), MEM sodium bicarbonate, MEM non-essential amino acids, MEM sodium pyruvate, fetal bovine serum (FBS), penicillin/streptomycin, Lipofectamine 2000 Reagent, DH5α cells are from Invitrogen (Oakville, ON, Canada). Anti-c-Src p[Y^{418}] and -STAT3 p[Y^{705}] polyclonal antibodies are from Biosource International (Camarillo, CA, USA). Anti-RalA monoclonal, anti-RalB polyclonal, anti-calmodulin monoclonal, anti-Ras monoclonal, anti-$p130^{Cas}$ monoclonal, IgG1 and anti-rabbit IgG antibodies are from Transduction Laboratories (Lexington, KY, USA). Anti-non-phosphorylated c-Src monoclonal antibody is from Cell Signaling (Pickering, ON, Canada). Anti-β1 monoclonal antibodies are from Sigma-Aldrich (St. Louis, MO, USA) and Upstate (Waltham, MA, USA). Recombinant EGF is from BD Bioscience (San Diego, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane, isopropylthio-β-D-galactopyranoside (IPTG) and Rapid DNA Ligation Kit are from Roche Diagnostics (Laval, QC, Canada). Nitrocellulose Trans-Blot Transfer Medium and horseradish peroxidase (HRP)-conjugated secondary antibodies are from Bio-Rad (Mississauga, ON, Canada). Matchmaker Two-Hybrid System 3, pGBKT7, pGADT7, pGBKT7[murine p53], pGBKT7[lamin C], pGADT7[SV40 large T-antigen], pCL1 expression plasmids, AH109 and Y187 yeast strains, Yeast Transformation Kit, all yeast media, X-α-Gal are from Clontech (San Diego, CA, USA). W7•HCl, W5•HCl and Go6976 are from Calbiochem (La Jolla, CA, USA). Protein A/G Plus-agarose is from Santa Cruz Laboratories (Santa Cruz, CA, USA). TnT Coupled Reticulocyte Lysate

System, amino acid mixture minus methionine, Plasmid Miniprep and Maxiprep Purification Systems are from Promega (Madison, WI, USA). [^{35}S]Methionine (sp. activity >1000 Ci/mmol), Kodak XOMAT-AR and BIOMAX-MR film, Enhanced Chemiluminescent (ECL) reagents, Sepharose 4B, calmodulin-Sepharose 4B are from Amersham Pharmacia Biotech (Montreal QC, Canada). Klenow fragment of DNA polymerase 1 is from Promega (Madison, WI, USA) and New England Biolabs (Pickering, ON, Canada). Quick Ligase Kit is from New England Biolabs (Pickering, ON, Canada). RPM Yeast Plasmid Isolation Kit was from Qbiogene Inc. (Carlsbad, CA, USA). Restriction enzymes were from Promega (Madison, WI, USA). Gibco BRL (Oakville, ON, Canada) and New England Biolabs (Pickering, ON, Canada). BL21-CodonPlus RIL cells are from Stratagene (La Jolla, CA, USA). A7r5 are from ATCC (Manassas, VA, USA). pRK-5 is from BD PharMingen (San Diego, CA, USA). AD202 cells were kindly supplied by Dr. N. Whitehead (Yale University, Boston, MA, USA). HeLa and MCF7 cells were a kind gift from Dr. G. Arthur (University of Manitoba, Winnipeg, MB, Canada). Enriched canine sarcolemmal membrane preparation (CSM) was a kind gift from Dr. G. Pierce (St Boniface General Hospital Research Centre, Winnipeg, MB, Canada). Respectively, plasmid constructs pGEX4T2[RIP1], pBF[CaM], pCRII[β1 subunit of Na^+/K^+ATPase], pSSRαII[p130^{Cas}] and pFLAG-CMV2[RalGPS1B] were generously provided by Dr. R. A. Weinberg (Whitehead Institute, Cambridge, MA, USA), Dr. J. P. Adelman (Oregon Health Sciences University, Portland, OR, USA), Dr. D. Bok (UCLA School of Medicine, Los Angeles, CA, USA), Dr. K. Vouri (The Burnham Institute, La Jolla, CA, USA), and Dr. L. Quilliam (Indiana University, Indianapolis, IN, USA). Plasmids pRK-5[RalAV23], pRK-5[RalBV23] and pRK-

5[RalBN28] were kind gifts from Dr. M. White (University of Texas, Dallas, TX, USA). All other chemicals and biologicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.2 Methods

4.2.1 Plasmid Constructs

pGBKT7[RalA]. pUC219[RalA] was restricted with HindIII and the resulting 900 base pair fragment containing RalA filled in to create blunt ends with Klenow fragment of DNA polymerase I. The RalA cDNA was ligated into Sfi1-restricted, Klenow-trimmed, dephosphorylated pGBKT7 to create the pGBKT7[RalA] construct.

5'....GCC A - **AG CTT ATG....**3'

pGBKT7 **RalA**

pGBKT7[RalA]

pGBKT7[RalB]. pLEX10[RalB] was restricted with BamH1 (at 5' end of RalB) and Sal1 (at 3' end of RalB), the resulting 621 base pair RalB fragment blunted with Klenow fragment, and ligated into pGBKT7 as above to create the pGBKT7[RalB] construct.

5'....GCC A - **GA TCC GGC GGT ATG....**3'

pGBKT7 **RalB**

pGBKT7[RalB]

pGADT7[CaM]. pBF[CaM] was restricted with Sal1 (at 5' end of CaM) and BglII (at 3' end of CaM), the resulting ~700 base pair CaM fragment filled in with Klenow fragment, and the CaM DNA ligated into Sfi1-restricted, Klenow-trimmed, dephosphorylated pGADT7 to create the pGADT7[CaM] construct..

5'....ATG G - **TC GAC ATG....**3'

pGADT7 **CaM**

pGADT7[CaM]

pGBKT7[RalA$_{1-264}$]. To cleave RalA roughly in half, pUC219[RalA] was restricted with HindIII and the resulting full length RalA fragment restricted with NlaIV. This generated two fragments (base pairs 1-264 and 265-621). The 5' half fragment of RalA was blunted with Klenow and ligated into SfiI-restricted, Klenow-trimmed, dephosphorylated pGBKT7 to create the pGBKT7[RalA$_{1-264}$] construct.

5'....GCC A - **AG CTT ATG....**3'

pGBKT7 **RalA$_{1-264}$**

pGBKT7[RalA$_{1-264}$]

pGBKT7[RalA$_{265-621}$]. The 3' half fragment of RalA was blunted with Klenow and ligated into Nde1-restricted, Klenow filled, dephosphorylated pGBKT7, to create the pGBKT7[RalA$_{265-621}$] construct.

5'...CAT A// **TCC...**3'

pGBKT7 **RalA$_{265-621}$**

pGBKT7[RalA$_{265-621}$]

pGBKT7[RalA$_{1-549}$]. To remove the C-terminal CaM BD from RalA, pUC219[RalA] was restricted with HindIII and Bbs1. HindIII isolated the full length RalA DNA, while BbsI restricted this fragment at base pair 549, eliminating the C-terminal CaM BD. This C-terminally deleted RalA cDNA was blunted with Klenow fragment and ligated into Sfi1-restricted, Klenow trimmed, dephosphorylated pGBKT7 plasmid to create pGBKT7[RalA$_{1-549}$] construct.

5'...GCC A - **AG CTT ATG...**3'

pGBKT7 **$RalA_{1-549}$**

pGBKT7[$RalA_{1-549}$]

pGBKT7[$RalA_{1-436}$]. pGBKT7[RalA] was restricted with Blp1, which restricted RalA at base pair 436, and with BamH1, which restricted the MCS 3' to the RalA insert, resulting in removal of base pairs 437-621 from the RalA insert. The restricted pGBKT7-$RalA_{1-436}$ DNA was then blunted with Klenow and self ligated to create the pGBKT7[$RalA_{1-436}$] construct.

pGBKT7[$RalB_{100-621}$]. To delete the first 99 5' base pairs from RalB, and thus eliminate the predicted CaM BD, pLEX10[RalB] was restricted with BamH1 and Sal1, and the resulting 621 base pair RalB fragment restricted with Hga1 to delete the first 99 5' base pairs. The resulting $RalB_{100-621}$ DNA fragment was blunted with Klenow fragment and ligated into Nco1-restricted, Klenow-blunted, dephosphorylated pGBKT7 to produce the pGBKT7[$RalB_{100-621}$] construct.

5' ....GCC ATG - **^{100}TTC ATG ...** 3'

pGBKT7 **$RalB_{100-621}$**

pGBKT7[$RalB_{100-621}$]

pGBKT7 [$RalB_{1-482}$]. pGBKT7[RalB] was restricted with Sma1. This construct has two Sma1 sites: at base pair 482 of RalB, and in MCS of pGBKT7, downstream of the RalB insert. The resulting pGBKT7-$RalB_{1-482}$ fragment was blunted with Klenow and self-ligated to create the pGBKT7[$RalB_{1-482}$] construct.

pGBKT7[$RalB_{100-486}$]. pGBKT7[$RalB_{100-621}$] was restricted with Sma1, and the resulting pGBKT7-$RalB_{100-486}$ fragment self-ligated to create the pGBKT7[$RalB_{100-486}$] construct.

pGBKT7[RalB$_{1-316}$]. To cut the RalB cDNA roughly in half, pGBKT7[RalB] was restricted with EcoR1. This construct has two EcoR I sites, at base pair 316 of RalB and in the MCS downstream of the RalB insert. The resulting pGBKT7-RalB$_{1-316}$ fragment was self-ligated to create the pGBKT7[RalB$_{1-316}$] construct.

pGBKT7[RalB$_{317-621}$]. The 3' 317-621 base pair half of RalB, produced by the above restriction of pGBKT7[RalB] with EcoR1, was ligated into EcoR1-restricted pGBKT7 to produce the pGBKT7[RalB$_{317-621}$] construct.

5'...GCC G**AA TTC AGG...**3'

pGBKT7 **RalB$_{317-621}$**

pGBKT7[RalB$_{317-621}$]

pGADT7[β1 subunit of Na^+/K^+ATPase]. The construct pCRII[β1 subunit of Na^+/K^+ATPase] was restricted with EcoR1 and the resulting β1 subunit of Na^+/K^+ATPase (β1) insert blunted with Klenow fragment. This cDNA was ligated into Sfi1-restricted, Klenow-trimmed and dephosphorylated pGADT7 expression plasmid to produce the pGADT7[β1] constructs. The β1 cDNA has a thymine residue inserted just before the ATG start codon to keep it in-frame.

5'...GCC ATG GCC A//**AA TTC GCC CTT ATG...**3'

pGADT7 or pGBKT7 **β1 Na^+/K^+ATPase**

pGBKT7/pGADT7[β1 Na^+/K^+ATPase]

pGEX-4T-2[β1]. The construct pCRII[β1] was restricted with EcoR1 and the resulting β1 insert blunted with Klenow fragment. This cDNA was ligated into Sal1-restricted, Klenow-blunted and dephosphorylated pGEX-4T-2 (Pharmacia) expression plasmid to produce the pGEX-4T-2[β1] construct.

5'...GGG TCG A//**AA TTC GCC CTT ATG...**3'

pGEX-4T-2 **β1 Na^+/K^+ATPase**

pGEX-4T-2[β1 Na^+/K^+ATPase]

pGEX-4T-2[Ral binding domain motif of RIP1]. The construct pGEX-4T-2[RIP1] was restricted with Xho1 and BstB1, creating a 1661 base pair fragment that includes the first 1385 base pairs of RIP1. This fragment was restricted with AflIII, generating a 299 base pair fragment of RIP1, from base pairs 1087 (AflIII) to 1385 (BstB1), which we termed RIP1 Ral BD (RRBD). The actual binding domain (BD) of Ral encompasses base pairs 1169-1331 of RIP1. The Ral BD motif of RIP1 base pairs 1087-1385) was blunted with Klenow fragment and ligated into Sma1-restricted, Klenow-blunted and dephosphorylated pGEX-4T-2 expression plasmid to produce the pGEX-4T-2[Ral BD motif of RIP1] construct (pGEX-4T-2[RRBD]).

5'...GGA ATT CCC // **CAT GTG...**3'

pGEX-4T-2 **Ral BD of RIP1**

pGEX-4T-2[Ral BD motif of RIP1]

4.2.1.1 Verification of plasmid[cDNA] constructs

All plasmid[cDNA] constructs were sequenced at University of Manitoba, Dept. of Cell Biology, to verify that the desired cDNA inserts were subcloned and were in-frame in the correct orientation. Prior to this, several DH5α *E. coli* colonies putatively containing each plasmid construct were screened for the presence of cDNA inserts and for determining the orientation by restriction enzyme analysis, as described below.

pGBKT7[RalA], -[$RalA_{1-265}$], -[$RalA_{1-436}$], -[$RalA_{1-549}$] constructs. To verify that the full length and deletion RalA construct fragments were ligated in the correct orientation

into pGBKT7, both HindIII and EcoRI restriction enzymes were used. (i) *HindIII.* pGBKT7 has three HindIII sites at base pairs 738, 1606 and 6544. RalA has no HindIII sites, but one was created at the 5' ligation site. HindIII-restricted pGBKT7[RalA] constructs therefore produced the expected four bands on agarose gel electrophoresis (AGE): 312 base pairs of plasmid plus length of RalA fragment, and plasmid bands of 556, 1494 and 4938 base pairs. (ii) *EcoRI.* pGBKT7[RalA] constructs have two EcoR1 sites: (i) at base pair 28 of RalA cDNA, and (ii) in the multiple cloning site (MCS) immediately 3' to the RalA insert. The restriction digest therefore produced the expected two bands of sizes equivalent to the insert minus 20 base pairs, plus 7.8 Kb plasmid band.

pGBKT7[RalA$_{265-621}$]. This construct has one BlpI site at base pair 436 of the RalA insert and three HindIII sites in the plasmid at base pairs 738, 1606 and 6544. Double restriction with these two enzymes therefore produced the expected four fragments of 716 (includes first 5' 171 base pairs of insert), 787 (includes remaining 3' 464 base pairs of the insert), 1500 and 5000 base pairs (both plasmid fragments).

pGBKT7[RalB]. This construct has two Sma1 sites; (i) at base pair 485 of RalB, and (ii) in MCS 10 base pairs immediately 3' to RalB. The correct construct produced two DNA fragments on Sma1 restriction and AGE; (i) 0.22 Kb (3' RalB), and (ii) 7.8 Kb (first 5' 485 base pairs of RalB plus pGBKT7).

pGBKT7[RalB$_{1-486}$]. This construct, being derived from pGBKT7[RalB], was automatically in-frame. To verify the insert was present, it was restricted with HindIII, which produced the three expected bands of 1376 (containing the RalB$_{1-486}$ fragment), 1494 and 4938 base pairs (plasmid DNA).

pGBKT7[RalB$_{316-621}$], pGBKT7[RalB$_{1-316}$], pGBKT7[RalB$_{1-482}$]. These constructs were restricted with HindIII to verify an insert was present i.e. presence of a DNA band containing the insert in frame with the 868 plasmid fragment, plus the 1494 and 4938 base pair plasmid DNA bands.

pGADT7[CaM]. This construct has four Pst1 sites: (i) at base pair 144 of CaM, and (ii) at base pairs 13, 2041 and 4511 of pGADT7. The correct construct produced four DNA fragments on Pst1 restriction and AGE; (i) 2.1 Kb (3' 144 base pairs of CaM plus 1969 bases of pGADT7 immediately 5' to CaM, (ii) 0.366 Kb (5' 306 base pairs of CaM plus 60 bases of pGADT7 immediately 3' to CaM, and (iii) 2.47 Kb and (iv) 3.5 Kb, both fragments of pGADT7.

Analysis of library clone pACT2[β1]. We used three different restriction enzymes, BglII, Nco1, Nde1. *BglII.* pACT2 has two BglII sites flanking the MCS, at base pairs 4982 and 5078. β1 has no BglII sites. Therefore BglII removes the insert from the pACT2[β1] construct, with 81 base pairs of the plasmid joined to the 5' end. A full length β1 insert would thus produce a DNA band of 986 base pairs with BglII (β1 cDNA is 905 base pairs). Results showed an insert of about 900 base pairs. Therefore, depending on how much 3' untranslated region is present, the cloned β1 has at least 100 base pairs missing. *Nco1.* There are two Nco1 sites in the pACT2[β1] construct, at base pairs 800 of β1 and in the MCS at 5018, 21 base pairs 5' to the β1 insert between EcoR1 (4997) and Xho1 (4988). This should produce two DNA bands of 821 (contains first 800 5' nucleotides) and 8.2 Kb (plasmid plus rest of β1). The library clone showed a truncated band of about 500-600 base pairs plus the plasmid band, suggesting the cloned β1 cDNA is missing about 300 5' base pairs. *Nde1.* There are four Nde1 sites in pACT2[β1] at base

pairs 300 of β1, and at 235, 5026 and 6131 of the plasmid. Nde1 should produce bands of 330 (contains first 300 5' nucleotides of β1), 6353 (contains remaining β1 nucleotides plus plasmid), and 1105 and 2204, both plasmid fragments. The library construct showed bands of 1105, 2204 and about 5000 base pairs. This suggests the Nde1 site in β1 is missing, and that therefore the β1 clone is missing at least 300 5' base pairs. *Nde1/Xho1.* To verify that the base pairs are missing from the 5' region of β1, the pACT2[β1] construct was restricted simultaneously with Xho1 and Nde1. The construct with full length β1 should result in 5 DNA bands of 1105, 2204 and 4753 (all plasmid DNA bands), plus 600 (3' β1) and 331 (5' β1) base pairs. Results demonstrated 3 plasmid bands plus one more of about 800 base pairs. Combined with the above Nde1 and BglII restriction results, this suggests that clone β1 has at least 300 nucleotides missing from the 5' end, and has some 200 base pairs of 3' UTR. Subsequent full sequencing determined that the clone pACT2[β1] cDNA was missing the first 351 5' base pairs.

pGADT7[β1]. *Nco1.* pGADT7[β1 Na^+/K^+ATPase] has three Nco1 sites: at base pair 800 of β1, and at base pairs 1935 and 1977 of pGADT7. Nco1 results in three fragments of 805 (includes first 800 5'base pairs of β1), and 8058 and 42 base pairs (pGADT7). *Nde1.* pGADT7[β1] has two Nde1 sites: at base pair 300 of β1, and at base pair 1971 of pGADT7. Nde1 restriction results in two fragments of 311 (first 300 5' base pairs of β1), and 8594 base pairs (605 3' base pairs of β1 plus pGADT7).

pGEX-4T-2[β1]. *BglI.* pGEX-4T-2[β1] has three Bgl1 sites at base pair 179 of β1, and base pairs 2041 and 4684 of the plasmid. Bgl1 restriction results in 3 fragments of 1415 (includes the first 179 5' base pairs of β1), 1817 (includes the remaining 726 3' base pairs of β1), and 2643 (plasmid fragment) base pairs. *AlwN1.* pGEX-4T-2[β1] has two AlwN1

sites at base pair 765 of β1, and base pairs 2639 of the plasmid. AlwN1 restriction results in two fragments of 4076 (includes the first 765 5' base pairs of β1), and 1829 (includes the remaining 765 3' base pairs of β1) base pairs.

pGEX-4T-2[RIP1 BD of RalA]. *Sac1*. pGEX-4T-2[RRBD] has one Sac1 restriction site at base pair 1095 of RIP1. Sac1 restriction results in a linear fragment that was sequenced to confirm correct orientation of a RIP1 BD of RalA insert.

pGEX-4T[RIP1]. Nco1. pGEX4T[RIP1] construct has two Nco1 sites at base pairs 200 and 1492 of the RIP1 insert. Nco1 thus produced two bands of 1292 (RIP1 fragment between base pairs 200 and 1492), and 5654 base pairs (plasmid plus rest of RIP1).

4.2.2 *In Vitro* Transcription/Translation

To verify that the pGBKT7[RalA], pGBKT7[RalB], pGBKT7[deletion constructs of RalA and RalB], pGADT7[β1 Na^+/K^+-ATPase] and pGADT7[CaM] constructs were in frame and could produce appropriate protein product, which were subsequently used for *in vitro* binding assays, all constructs and empty plasmids as controls were subjected to *in vitro* transcription/translation using the "TnT Coupled Reticulocyte Lysate System" in the presence of amino acid mixture minus methionine plus [^{35}S]methionine (>1000 Ci/mmol at 10 mCi/ml). The proteins produced would be the Ral proteins fused to c-Myc, and CaM and β1 fused to HA epitope tags. Five percent of the TnT reactions were subjected to 13% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel fixed for 30 minutes in fixing solution (50% methanol, 10% glacial acetic acid, 40% water), soaked for 10 minutes in a pre-drying solution (7% acetic acid, 7%

methanol, 1% glycerol), vacuum dried for 1 hour at 80°C, and exposed on Kodak X-OMAT-AR or Biomax MR film for 8-16 hours at –70°C.

4.2.3 Yeast Two-Hybrid (Y2H) Assay

Clontech's "Matchmaker Two-Hybrid System 3" was used throughout. RalA and RalB cDNAs, and their deletion constructs, were subcloned in-frame onto the 3' end of the GAL4 transcription factor binding domain (BD) in the pGBKT7 expression plasmid. Upon transformation into yeast strain AH109, expression of BD[Ral] fusion proteins occurs under control of a truncated ADH1 promoter. CaM and β1 cDNAs were subcloned in-frame onto the 3' end of the GAL4 transcription factor activation domain (AD) in the pGADT7 expression plasmid. Upon transformation into yeast strain AH109, expression of AD[CaM] and AD[β1] fusion proteins occurs under control of the full length ADH1 promoter. In addition, pGBKT7 and pGADT7 are induced to synthesize tryptophan and leucine, respectively. Following small and large scale yeast transformation protocols (Clontech), using simultaneous and sequential transformation procedures, the transformed AH109 yeast cells, which are auxotrophic for leucine and tryptophan and which contain reporter genes HIS3, ADE2, LacZ and MEL1 under the control of separate promoters but all activated by GAL4 transcription factor, were spread onto minimal synthetic dropout (SD) medium agar plates lacking histidine, adenine, leucine and tryptophan (SD/-HALT). To check for activation of the MEL1 gene and expression of α-galactosidase, X-α-Gal was spread onto the surface of each plate (100 μl or 200 μl of 2 mg/ml X-α-Gal in dimethylformamide on 10 cm and 15 cm plates, respectively). α-Galactosidase digests X-α-Gal to produce a blue by-product. The plates

were incubated at 30°C for 2-10 days. Any blue colonies were re-streaked at least two times onto fresh SD/-HALT/+X-α-Gal plates to ensure propagation of correct phenotype. As positive controls, AH109 was transformed with (i) pCL1, which contains the complete GAL4 transcription factor, and spread on to SD/-Leu/+X-α-Gal plates, and (ii) pGBKT7[murine p53] and pGADT7[SV40 large T-antigen], which were spread onto SD/-Trp/-Leu/+X-α-Gal plates. As negative controls, AH109 was transformed with (i) pGBKT7[lamin C] and pGADT7[T-antigen], and (ii) pGBKT7[lamin C] and pGADT7[CaM). The transformed cells were spread onto SD/-Trp/-Leu/+X-α-Gal plates. To ensure the BD and AD constructs did not activate the MEL1 gene autonomously, AH109 yeast strain was transformed with pGBKT7[Rals], or pGADT7[CaM] and pGADT7[β1], and spread onto SD/-Trp /+X-α-Gal (Ral constructs) or SD/-Leu/+X-α-Gal (CaM and β1 constructs) plates.

4.2.4 Yeast Mating

A large fresh (less than 2 months old) colony of AH109 transformed with pGBKT7[RalA], -[RalB], and -[Ral deletion constructs] were mated with a fresh colony of Y187 transformed with pGADT7[CaM] or -[β1], as per Clontech's large and small scale protocols. Mated incubates were spread onto SD/-HALT/+X-α-Gal plates and incubated at 30°C for 2-10 days. Any blue colonies were re-streaked at least 3 times onto fresh SD/-HALT/+X-α-Gal plates to ensure correct phenotype.

4.2.5 Screening of Human Testis cDNA Expression Library

A large fresh (<2 months old) colony of AH109 yeast cells transformed with pGBKT7[RaIB] was mated with 1 ml of the testis cDNA expression library subcloned into pACT2 expression vector, as per Clontech's protocol. The total mating mixture of cells was spread over fifty 15cm SD/-HALT/+X-α-Gal plates and incubated at 30°C for 2-10 days. Any blue colonies were subjected to further analysis.

4.2.6 Isolation of Yeast Plasmids

A colony each of positive clones #1, 2, 3, 4, 7, and 8 from the human testis cDNA library screening was added to 10 ml SD/-HALT medium and incubated for 1-2 days at 30°C and 200 rpm. Plasmid and genomic DNA was isolated from the yeast using the RPM Yeast Plasmid Isolation Kit. The DNA was eluted in 100 μl double-distilled water, and 25 μl of this used to transform 100 μl of DH5α *E. coli* cells. All of the transformed cells for each clone were spread onto an LB/ampicillin (100 mg/ml) plate to select only for pACT2[library clones]. DNA was isolated from colonies by plasmid minipreps, and the DNA was restricted with BglII to verify a cDNA insert was present. There are two BglII sites in the pACT2 plasmid; at the 5' and 3' ends of the MCS. DNA from several colonies of each clone 1, 2, 3, 4, 7 and 8 were restricted with BglII, and all DNA from each clone showed identical cDNA inserts by length.

4.2.7 Sequencing of Testis Library cDNAs

The positive human testis cDNA clones inserted in pACT2 plasmids, were sequenced at the DNA Sequencing Laboratory, University Core DNA and Protein

Services, University of Calgary, and at the Dept of cell Biology, University of Manitoba, Winnipeg. Clones 4 and 8 were sequenced at Calgary using the Gal4 3' AD sequencing primer, and at Winnipeg using the 5' AD sequencing primer. The resulting sequences were entered into BLASTN to identify significant alignments.

4.2.8 Western blot of BD-cMyc-RalA, -RalB, and AD-HA-CaM and -β1 from Transformed Yeast

To verify that the BD- and AD-fusion proteins were expressed in yeast, the BD-RalA and BD-RalB fusion proteins, plus the AD-CaM and AD-β1 fusion proteins were extracted from the transformed AH109 and Y187 yeast cells, protocol as per Clontech's urea/SDS method. Five μl of each protein sample was added to SDS sample buffer, heated at 100°C for 2 minutes and separated by 13% SDS-PAGE. The proteins were transferred to PVDF membrane and immunoblotted with the appropriate antibodies to RalA, RalB, CaM and β1, followed by ECL and autoradiography on Kodak X-OMAT-AR film.

4.2.9 *In Vitro* Binding Assays

Using the TnT Coupled Reticulocyte Lysate System in the presence of amino acid mixture minus methionine plus [^{35}S]methionine, [^{35}S]Met-labelled RalA, RalB and Ral deletion constructs were transcribed and translated *in vitro* from 1 μg of appropriate pGBKT7[Ral] construct, and [^{35}S]Met-labelled CaM and β1 were transcribed and translated *in vitro* from 1 μg pGADT7[CaM] or pGADT7[β1], respectively,. The binding reactions were carried out based on the protocol of Jullien-Flores *et al.,* (1995). Briefly,

50 µl of Sepharose-CNBr-coupled proteins and control Sepharose beads as negative control, or 50 µl of GSH-agarose-coupled GST-fusion proteins and GST as negative control, were washed twice in ice-cold *in vitro* binding buffer {20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 200 mM NaCl, 0.5% Nonidet P-40, and 1 mM 4-[(2-aminoethyl)]-benzene sulfonyl fluoride (AEBSF)} and incubated overnight at 4°C with 10 µl of appropriate *in vitro* transcribed/translated [^{35}S]methionine-labelled protein in 100 µl binding buffer. After sedimentation of the beads, the supernatant was removed and the beads were washed three times with ice-cold binding buffer containing 1 mM dithiothreitol. The bound proteins were recovered by boiling the beads in 1× Laemmli's sample buffer, and separated by 13% SDS-PAGE. The gel was fixed for 1 hour in 50% methanol, 10% acetic acid and 40% water, washed twice in double-distilled water for 15 minutes each, treated for 1 hour with 1 M sodium salicylate (pH 7.0), and soaked in pre-drying buffer (7% methanol, 7% acetic acid, 1% glycerol) for 10 minutes. The gel was dried and the presence of [^{35}S]Met-labelled proteins was detected by autoradiography at -70°C for 1-7 days. All binding reactions were repeated in reverse, in which proteins that were [^{35}S]Met-labelled were now instead attached to Sepharose or agarose beads, and proteins that were attached to Sepharose or agarose beads were now instead [^{35}S]Met-labelled. In all assays, the binding buffer was used with no additions, or with 0.5 mM $CaCl_2$ or 5 mM EGTA/EDTA added, as indicated.

4.2.10 Platelet Washing

Partially purified human platelets obtained from the Canadian Blood Services (Winnipeg, MB, Canada) or freshly drawn human platelets were gently rocked and

treated for 30 minutes with 0.1 volume ACD buffer (1.5% citric acid, 2.5% trisodium citrate, 2% glucose) (Wolthuis *et al.*. 1998b). The platelets were centrifuged at 600 x g for 15 minutes to remove any remaining erythrocytes. The supernatant containing the purified platelets was centrifuged at 1,000 x g for 15 minutes, and the platelet pellets separated from remaining erythrocytes by washing in 10 mM N-(2-hydroxy ethyl)piperazine-N'-(2-ethane) sulfonic acid (HEPES)/Tyrode buffer (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 5 mM glucose), or buffered saline (10 mM HEPES, pH 7.4, 145 mM NaCl, 5 mM KCl, 10 mM Glucose, 1mM $MgSO_4$, 0.5 mM EGTA). The platelets were resuspended in 5-10 ml HEPES/Tyrode buffer, and stored at –20°C, or kept at room temperature for 30 minutes to ensure a resting state, and later treated with thrombin.

4.2.11 Fractionation of Platelets

Purified platelets were suspended in HEPES/Tyrode buffer or fractionation buffer (20 mM HEPES, pH 7.4, 200 mM KCl, 1 mM $MgCl_2$, and 1 mM phenylmethylsulfonylfluoride [PMSF]), and lysed by sonication. Proteins were centrifuged at 100,000 x g for 2 hours at 4°C. The supernatant containing cytosol was used as the source of endogenous CaM. The cytosol was stored at –80°C. The pellet was resuspended in solubilization buffer (20 mM HEPES, pH 7.4, 200 mM KCl, 1 mM $MgCl_2$, 20% glycerol, 0.55% Triton X-100, 1 mM PMSF), and incubated for 1 hour at 4°C and centrifuged at 50,000 x g for 30 minutes. The supernatant containing solubilized particulate proteins were divided into aliquots and stored at -80°C. To obtain total platelet lysate, purified platelets were suspended and sonicated in buffer containing HEPES, pH

7.4, 200 mM KCl, 1 mM $MgCl_2$, 0.55% Triton X-100, 20% glycerol, and centrifuged at 100,000 x g for 2 hours at 4°C. Total platelet lysate was stored at -80°C.

4.2.12 Expression of GST-Fusion Proteins in *E. coli*, and Preparation of GSH-Agarose-Coupled GST-Fusion Proteins

Before fusion proteins were expressed, all pGEX-4T-2[RRBD], pGEX-4T-2[β1] and pGEX-2T[Ral] plasmid constructs were isolated from each bacterial colony by plasmid mini-prep, and the DNA subject to restriction enzyme analysis to ensure the appropriate cDNA was present in the bacteria. To help verify that glutathione S-transferase (GST)-fusion proteins were in-frame and could be expressed for subsequent use in binding reactions, large and small scale protocols for protein expression were used. For large scale, an aliquot of previously frozen transfected bacteria (-70°C) was grown in 5 ml of LB/ampicillin (50 μg/ml) broth for 16 hours at 37°C and 200 rpm shake. The incubate was added to 500 ml LB/ampicillin (LB/amp) and incubated for a further 16 hours at room temperature and 200 rpm shake. For small scale, a 100 μl aliquot of previously frozen transfected bacteria were grown in 5 ml of LB/amp (50 μg/ml) for 8 hours at 37°C and 200 rpm shake, followed by incubation at room temperature overnight. To stimulate GST-fusion protein expression, 0.5 mM (1 mM for small scale) IPTG was added to the incubates for 2-4 hours at room temperature, after which the cells were pelleted at 6,000 rpm for 15 minutes (14,000 x g for 5 seconds for small scale) and frozen at -20°C or used immediately. The bacterial cells were resuspended in 20 ml (1.5 ml small scale) NETT buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100) and homogenized. To the homogenates were added 40 mg (4 mg small

scale) of lysozyme and 1 mM AEBSF, and the total volume corrected to 40 ml (1.5 ml small scale) with NETT buffer. After incubating at room temperature for 15 minutes, the cells were disrupted by sonication (3 pulses of 15 seconds each) and then centrifuged at 16,000 x g for 30 minutes. The supernatant was used immediately or frozen at -80°C after the addition of 10% glycerol. The total supernatant was mixed with 0.75 ml (75 µl small scale) of water-washed glutathione (GSH)-agarose beads (0.1 g per 1.5 ml NT buffer [20 mM Tris-HCl, pH 7.5, 100 mM NaCl]) and the mixture rocked gently at 4°C for 30 minutes. The GSH-agarose-coupled GST-fusion protein beads were pelleted at 2,000 rpm for 10 minutes (10,000 rpm for 1 minute small scale) and washed three times in NETT buffer, followed by two washes in NT buffer. For large scale, the beads were suspended 1:1 v/v in Dulbecco's phosphate buffer plus 10% glycerol and stored at -20°C. For small scale, the beads were resuspended in 25 µl Laemmli's buffer and subject to SDS-PAGE. The gels were stained with Coomassie Blue to highlight expressed GST-fusion proteins. Alternatively, 10 µl of the agarose-fusion protein beads were added to 15 µl of Laemmli buffer, subjected to SDS-PAGE and western blotting performed with appropriate antibodies. GST-RalA and GST-RalB fusion proteins were expressed in DH5α cells, GST-RRBD in AD202 cells made competent by the rubidium chloride method (as per Promega protocol), and GST-β1 in BL21-CodonPlus RIL cells.

4.2.13 Thrombin-Cleavage of GST-Fusion Proteins

GSH-agarose-coupled GST-RalA, –RalB or -β1-fusion proteins were resuspended 1:1 in phosphate buffered saline (PBS) (0.02 M NaH_2PO_4, 0.3 M NaCl, pH 7.4). To these were added 10 µl of thrombin (50 NIH units/ml) and the mixture gently rocked overnight

at room temperature. After incubation, the beads were pelleted and 10µl each of supernatant (cleaved proteins in PBS) and beads were subject to SDS-PAGE to determine efficiency of thrombin cleavage. The supernatant containing the proteins was stored at -20°C or used immediately.

4.2.14 Coupling of Recombinant Proteins to Sepharose-CNBr Beads

Thrombin-cleaved RalA, RalB or β1 proteins were mixed 0.5:1 v/v with 0.1-0.3 g of cyanogen bromide (CNBr)-activated Sepharose beads that had been washed in 1 mM HCl (15 times bead volume), and the mixture gently rocked overnight at 4°C. The beads were pelleted, the supernatant decanted, 1.0 ml of 10 mM Tris-HCl, pH 7.5 added, and the mixture rocked gently at 4°C for 3 hours. The beads were pelleted and washed in 1.0 ml of 10 mM Tris-HCl, pH 8-9, followed by washing in 1.0 ml of 100 mM acetate buffer, pH 4.0. This washing process was repeated 5 times, after which the beads were recovered and stored in 20% ethanol at 4°C.

4.2.15 Pull-Down of Endogenous Ral (or β1) with GST-β1 (or GST-Ral), and Pulldown of Ral-GTP Using GST-RRBD, in Human Platelets

Experimental procedures were based on the protocol of Wolthuis *et al.* (1998b). For the Ral-GTP/RRBD interactions, freshly prepared platelets were divided into 500 µl aliquots, and stimulation with 0.2 U/ml thrombin was performed at 37°C without stirring for 10 and 90 seconds. W7 and W5 (50 µM) were added 10 minutes before agonist treatment. Platelets were lysed (2:1 v/v) in ice-cold 3 x Ral buffer (final concentration: 10% glycerol, 1% Igepal CA-630, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2.5 mM

$MgCl_2$, 1 mM AEBSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 μM pepstatin A, 1 mM benzamidine) for 30 minutes at 4°C with gentle rocking. Lysates were clarified by centrifugation at 16,000 x g at 4°C for 10 minutes, and 0.75 ml of the supernatant of each sample incubated with 50 μl of GST-RRBD precoupled to GSH-agarose beads. Samples were incubated for 1 hour on a rocker at 4°C. Beads were collected by centrifugation (9,000 rpm for 1 minute) and washed 4 times in Ral buffer. Beads were resuspended in 50 μl 1 x Laemmli buffer, boiled and 25 μl of this subjected to SDS-PAGE in duplicate and western blotting performed with anti-RalA monoclonal (1:5000) and anti-RalB polyclonal (1:125) antibodies.

For the β1/Ral interactions, outdated human platelets were prepared as above and 6 ml lysed 2:1 (v:v) with 3x Ral buffer. The supernatant (10 ml) was incubated with 300 μl of GST-β1 (or GST-RalA and –RalB) precoupled to GSH-agarose beads, and endogenous Ral (or β1) pulled down as described above. As a negative control, 1 ml of supernatant was incubated with GST-coupled GSH-agarose beads. Proteins boiled from the beads were subjected to SDS-PAGE and western blotting performed with anti-RalA and anti-RalB (or anti- β1) antibodies.

4.2.16 Pull-Down of Endogenous β1 (or Endogenous RalA and RalB) in Canine Sarcolemmal Membranes by Sepharose-CNBr-Coupled RalA and RalB (or Sepharose-CNBr-Coupled β1)

Liquid nitrogen-frozen enriched canine sarcolemmal membranes (CSM) suspended in 30% sucrose and 20 mM Tris-HCl, pH 7.0 were washed in PBS and the proteins solubilized in 500 μl radioimmune precipitation buffer (RIPA) (50 mM Tris-

HCl, pH 7.5, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM AEBSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin) at 4°C with gentle rocking for 1 hour. The supernatant was clarified by centrifugation at 16,000 x g for 10 minutes and the protein concentration determined by Bradford assay. Sepharose-CNBr-coupled RalA or RalB beads (50 μl), plus blocked Sepharose-CNBr beads as negative control, were washed twice in ice cold Ral buffer. The beads were resuspended in 500 μl of the buffer, 100 μg of the CSM supernatant was added, and the mixture rocked for 3 hours at 4°C. The beads were recovered by centrifugation, washed four times in Ral buffer, and resuspended in Laemmli's buffer. The proteins were boiled off the beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and probed with anti-β1 monoclonal antibody. In the reverse reaction, CSM were prepared as above. Sepharose-CNBr-coupled β1 beads (50 μl), plus Sepharose-CNBr beads as negative control, were incubated with 100 μg of the CSM supernatant as above, and subjected to 13% SDS-PAGE/western blotting with anti-RalA and anti-RalB antibodies.

4.2.17 Co-Immunoprecipitation of Ral and CaM in Human Platelets

Freshly drawn human platelets were prepared as above, divided into 1 ml aliquots and treated with 0.2 U/ml thrombin for 60 seconds at 37°C without stirring. The platelets were lysed by sonication with 1:1 v/v platelet buffer containing 316 mM NaCl, 2 mM EGTA, 20 mM Tris-HCl, pH 7.6, with protease inhibitors at a final concentration of 1 mM AEBSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM benzamidine, plus 1 mM Na-orthovanadate. After sonication at 4°C, the lysates were cleared by centrifugation (17,000 x g for 60 minutes), and the supernatants precleared with 20 μl Protein A/G Plus-

agarose and 0.2 μg of rabbit anti-mouse IgG1 with gentle rocking at 4°C for 30-60 minutes. The supernatants were cleared by centrifugation (16,000 x g for 1 minute), incubated with 5 μg/ml anti-CaM monoclonal antibody, and rocked at 4°C for 1 hour. Control platelet lysates were incubated with 5 μg/ml IgG1. Protein A/G Plus-agarose (30 μl) was then added, and the mixtures rocked at 4°C for 30-60 minutes. The beads were collected by centrifugation (16,000 x g for 1 minute), washed four times in TN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). The beads were resuspended in 25 μl Laemmli's buffer, boiled for 3 minutes, centrifuged at 17,000 x g for 4 minutes, and the proteins separated by 15% SDS-PAGE. Proteins were transferred to PVDF membranes and probed with anti-RalB polyclonal (1:125) antibody. The membrane was then stripped by heating to 50°C for 30 minutes in stripping buffer (62.5 mM Tris-HCl, pH 7.4, 2% SDS) containing 7.1 μl/ml of β-mercaptoethanol, and reprobed with anti-CaM monoclonal antibody to verify presence of CaM.

4.2.18 Cell Culture

A7r5 (rat aorta smooth muscle) and MCF7 (human breast adenoma) cells were maintained in DMEM supplemented with 1.5 g/l sodium bicarbonate, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (FBS). HeLa cells (human cervical adenocarcinoma) were maintained in MEM supplemented with 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum. All cells were incubated at 37°C in humidified air and 5% CO_2. Cells were seeded to 10 cm plates for treatments or 35 mm^2 plates for transfections. At about 80% confluence on 10 cm plates

the medium was changed to medium without serum for 24 hours (A7r5 and HeLa cells) or 5 days (MCF7 cells) to make the cells quiescent before indicated treatments.

4.2.19 Cell Culture Transfections

A7r5 and HeLa cells were seeded onto 35 mm^2 plates. When 90% confluent, the medium was replaced with medium lacking antibiotics for 24 hours. The medium was then replaced with medium lacking antibiotic and FBS, and the cells transfected with plasmid[cDNA] constructs as indicated using Lipofectamine 2000 Reagent, according to manufacturer's instructions.

4.2.20 Co-Immunoprecipitation of RalA and RalB and β1, and of RalB and c-Src, in HeLa Cells

HeLa cells were washed in Dulbecco's PBS, lysed by sonication in RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl) plus protease inhibitors, and put on ice for 30 minutes. The supernatants were cleared by centrifugation (17,000 x g for 10 minutes), precleared with 20 μl protein A/G Plus-Agarose for 30 minutes, and precipitated with 4 μg anti-β1, IgG2a, anti-RalB, or rabbit IgG for 1.5 hours at 4°C. The precipitates were then pulled down with 50 μl protein A/G Plus-Agarose for 1.5 hours, the beads collected by centrifugation and washed four times in RIPA buffer. The proteins were dissolved in Laemmli's buffer, boiled for 3 minutes, centrifuged for 4 minutes, separated by 13% SDS-PAGE, and transferred to PVDF membranes. The membranes were probed with anti-RalA (β1 and IgG2a precipitates), or

anti-β1 (RalB and rabbit IgG precipitates), or anti-c-Src (separate RalB and rabbit IgG precipitates) antibodies.

4.2.21 Detection of Active GTP-bound Ral Using GST-RRBD in Cultured Cells

Subconfluent, quiescent A7r5, HeLa and MCF7 cells were variably treated with 50 or 100 μM W7, 20 or 50 μM BAPTA-AM, 10 or 20 nM Go6976, 10 or 20 μM Rottlerin, or 5 or 10 μM Ro318220 for 30 minutes, or treated with 10 or 30 μM U73122 for 5 minutes before a 15 minute treatment with 10 ng/ml of recombinant EGF, or a 5 or 30 minute treatment with 1 μM PMA. HeLa and A7r5 cells were also treated with 1 or 100 μM, respectively of ouabain for 15 minutes, 2 hours or 24 hours, in some cases followed by a 15 minute treatment with 10 ng/ml of recombinant EGF. Some cells were transfected with pRK-5[RalB(N28)], pFLAG-CMV2[RalGPS1B] or pSSRαII[p130^{Cas}] constructs 24 hours before treatments. To detect levels of activated Ral, cells were washed twice in ice-cold Dulbecco's PBS, lysed in 0.5 ml of ice-cold Ral lysis buffer, sonicated briefly, and incubated for 30 minutes on ice. Lysates were clarified by centrifugation at 17,000 x g at 4°C for 10 minutes, and the supernatant of each sample incubated with 50 μl of GST-RRBD coupled to GSH-agarose beads (Clough *et al.*, 2002) to pull down GTP-bound Ral (Rodriguez-Viciana *et al.*, 1997). Samples were incubated for 1 hour on a rocker at 4°C. Beads were collected by centrifugation (12,000 rpm for 1 minute) and washed four times in Ral buffer. Beads were resuspended in 25 μl Laemmli loading buffer, boiled for 3 minutes, and centrifuged at 12,000 x g for 4 minutes. The proteins were separated by 13% SDS-PAGE, transferred to PVDF membranes, and western blotting performed with anti-RalA monoclonal and anti-RalB polyclonal

antibodies. Signals were detected using ECL. To ensure lysates contained equal protein concentrations, a 10 µl aliquot of each lysate was removed before beads were added, and was subjected to 13% SDS-PAGE/western blotting with anti-β-actin monoclonal antibody.

4.2.22 Detection of Expression of Transfected Proteins in Cultured Cells

Cells transfected with RalB(N28), RalA(V23), RalB(V23), $p130^{Cas}$ and FLAG-tagged RalGPS1B cDNAs, plus untransfected cells as controls, were washed twice in ice-cold PBS, lysed in 2 x Laemmli buffer, sonicated briefly, and incubated for 30 minutes on ice. Lysates were clarified by centrifugation at 17,000 x g at 4°C for 10 minutes, proteins separated by 7.5% ($p130^{Cas}$) or 13% (Ral, RalGPS) SDS-PAGE, transferred to nitrocellulose ($p130^{Cas}$) or PVDF (Ral, RalGPS) membranes, and total proteins detected using anti-RalA, -RalB, - $p130^{Cas}$ or -FLAG antibodies. Signals were detected using ECL.

4.2.23 Detection of Active Phosphorylated Forms of c-Src and STAT3

Subconfluent, quiescent A7r5 or HeLa cells were treated with 50 or 100 µM W7, 20 or 60 µM BAPTA-AM or 10 or 30 µM U73122 for 30 minutes before a 15 minute treatment with 10 ng/ml of recombinant EGF. Some cells were transfected with constitutively active [RalA(V23), RalB(V23)] or dominant-negative [RalB(N28)] Ral constructs before indicated treatments. Cells were washed twice in ice-cold Dulbecco's PBS, lysed in 0.75 ml of ice-cold 2 x Laemmli's buffer or RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% Igepal CA-630, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM AEBSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate, 1

mM sodium fluoride, 10 μM okadaic acid, 10 mM tetrasodium pyrophosphate), sonicated briefly, and incubated for 30 minutes on ice. Lysates were clarified by centrifugation at 17,000 x g at 4°C for 10 minutes, and 30 μl of the supernatant of each sample boiled for 3 minutes, centrifuged at 12,000 x g for 4 minutes, and the proteins separated by 13% SDS-PAGE. The separated proteins were transferred to PVDF membranes, the membranes immunoblotted with anti-c-Src p[Y^{418}] or -STAT3 p[Y^{705}] polyclonal antibodies.

4.2.24 Detection of Active Phosphorylated c-Src in Ouabain-Treated A7r5 and HeLa Cells

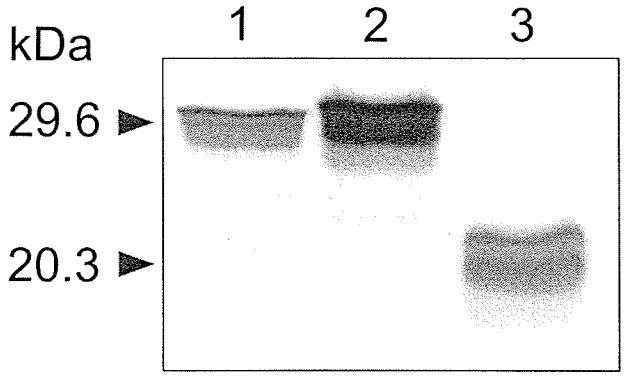
Quiescent, 90% subconfluent A7r5 or HeLa cells were transfected with RalB(N28) or RalA(V23), and 24 hours later treated for 5 minutes with 100 μM or 1 μM ouabain, respectively. Other A7r5 and HeLa cells were treated with 50 μM W7 before a 5 minute treatment with 100 μM or 1 μM ouabain, respectively. Cells were washed twice in ice-cold Dulbecco's PBS, lysed in 200 μl of modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% Igepal CA-630, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM AEBSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 μM okadaic acid, 10 mM tetrasodium pyrophosphate), sonicated briefly, and incubated for 30 minutes on ice. Lysates were clarified by centrifugation at 17,000 x g at 4°C for 10 minutes, and 30 μl of the supernatant of each sample boiled for 3 minutes, centrifuged at 12,000 x g for 4 minutes, and the proteins separated by 13% SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes. The membranes were probed with anti-c-Src p[Y^{418}] polyclonal antibodies, and signals detected by ECL.

5. RESULTS

5.1 Ral Interacts Directly with Calmodulin, and Requires Calmodulin for Activation

5.1.1 Full-Length and Deleted Ral and CaM cDNAs Undergo *In Vitro* Transcription/Translation from pGBKT7 and pGADT7 Expression Plasmids

We initially verified that full length and deleted Ral cDNAs were inserted in-frame onto the 3' end of the Myc epitope tag in the pGBKT7 expression plasmid, and that the CaM cDNA was inserted in-frame onto the 3' end of the HA epitope tag in the pGADT7 expression plasmid, which would enable the plasmid constructs to synthesize appropriate epitope-tagged Ral and CaM proteins. Therefore, the pGBKT7[Ral] and pGADT7[CaM] constructs plus empty plasmids as controls, were subjected to *in vitro* transcription/translation in the presence of [^{35}S]methionine. SDS-PAGE and autoradiography showed that full length RalA (Fig. 7, upper and lower panel, lane 1), RalB (Fig. 7, upper panel, lane 2, lower panel, lane 3), and CaM (Fig. 7, upper panel, lane 3), as well as truncated products $RalA_{1\text{-}549}$ (lacks the predicted C-terminal CaM BD) (Fig. 7, lower panel, lane 1), $RalB_{1\text{-}482}$ (lacks the C-terminal region equivalent to the RalA CaM BD) (Fig. 7, lower panel, lane 4), and $RalB_{100\text{-}482}$ (lacks both the predicted N-terminal [http:/calcium.oci.utoronto.ca/]) and C-terminal CaM BDs) (Fig. 7, lower panel, lane 5), were all translated *in vitro*. No proteins were translated from empty pGBKT7 and pGADT7 plasmids (results not shown). Extra bands seen in some lanes may be proteolytic degradation products, protein products being translated from other start sites, or background resulting from non-specific binding of the antibodies. These results help verify that all cDNAs were inserted in-frame into the appropriate expression vectors, and that the correct protein products are able to be expressed.



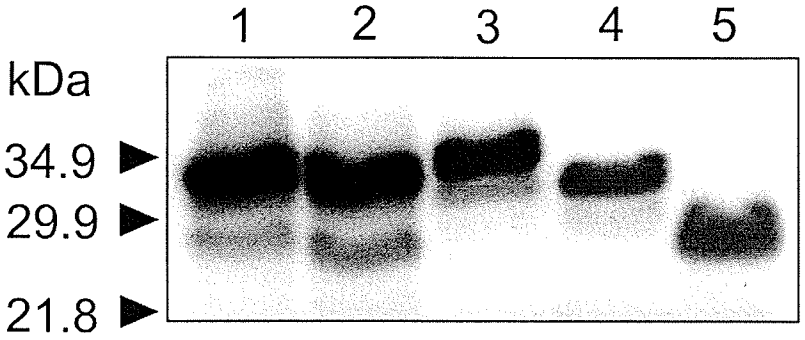


Figure 7

Fig. 7. Full length and deleted Ral and CaM cDNAs undergo *in vitro* transcription/translation from pGBKT7 and pGADT7 expression plasmids. Constructs pGBKT7[RalA], pGBKT7[RalB], pGBKT7[$RalA_{1-549}$], pGBKT7[$RalB_{1-482}$], pGBKT7[$RalB_{100-482}$] and pGADT7[CaM] underwent *in vitro* transcription/translation in the presence of [^{35}S]methionine as per Methods. Five per cent of the radiolabelled translated proteins were subjected to 13% SDS-PAGE and autoradiography to detect c-Myc/RalA (upper and lower panel, lane 1), c-Myc/RalB (upper panel, lane 2, lower panel, lane 3), HA/CaM (upper panel, lane 3), c-Myc/$RalA_{1-549}$ (lower panel, lane 2), c-Myc/$RalB_{1-482}$ (lower panel, lane 4), and c-Myc/$RalB_{100-482}$ (lower panel, lane 5).

5.1.2 Recombinant RalA and RalB and CaM Interact Specifically *In Vitro* in a Ca^{2+}-Dependent Manner

It has previously been shown that RalA has a C-terminal CaM BD (Wang *et al.*, 1997). To determine, for the first time, whether both RalB and RalA interact with CaM, an *in vitro* binding reaction was performed as described in Methods. Autoradiography showed that [^{35}S]Met-labelled RalA interacted with CaM-Sepharose in the presence of 0.5 mM $CaCl_2$ (Fig. 8A, Lane 1) at a significantly greater degree than in the presence of 5 mM EDTA (Fig. 8A, Lane 2). The interactions were specific because Sepharose-CNBr control beads did not bind [^{35}S]Met-labelled RalA (Fig. 8A, Lane 3). Extra bands seen in some lanes may be proteolytic degradation products, protein products being translated from other start sites, or background resulting from non-specific binding of the antibodies. In the reverse reaction, Sepharose-CNBr-coupled CaM BD of RalA (Fig. 8B, Lane 2) and recombinant RalB (Fig. 8B, Lane 3) formed complexes with [^{35}S]CaM. This Ral/CaM interaction was Ca^{2+}-dependent. CaM bound to Sepharose-CNBr-coupled CaM BD of RalA (Fig. 8C, Lane 2) and recombinant RalB (Fig. 8C, Lane 3) in the presence of 0.5 mM $CaCl_2$ to a much greater extent than in the presence of 5 mM EDTA (Fig. 8C, Lanes 5 and 6, respectively). The interactions were specific because Sepharose-CNBr control beads did not bind CaM (Fig. 8B and 8C, Lane 1). These results indicate that recombinant Ral and CaM proteins interact in a Ca^{2+}-dependent manner.

5.1.3 Endogenous CaM and Ral Co-Immunoprecipitate in Human Platelets

Co-immunoprecipitation experiments were performed to establish whether endogenous Ral and CaM form complexes in platelets. We have previously shown that in

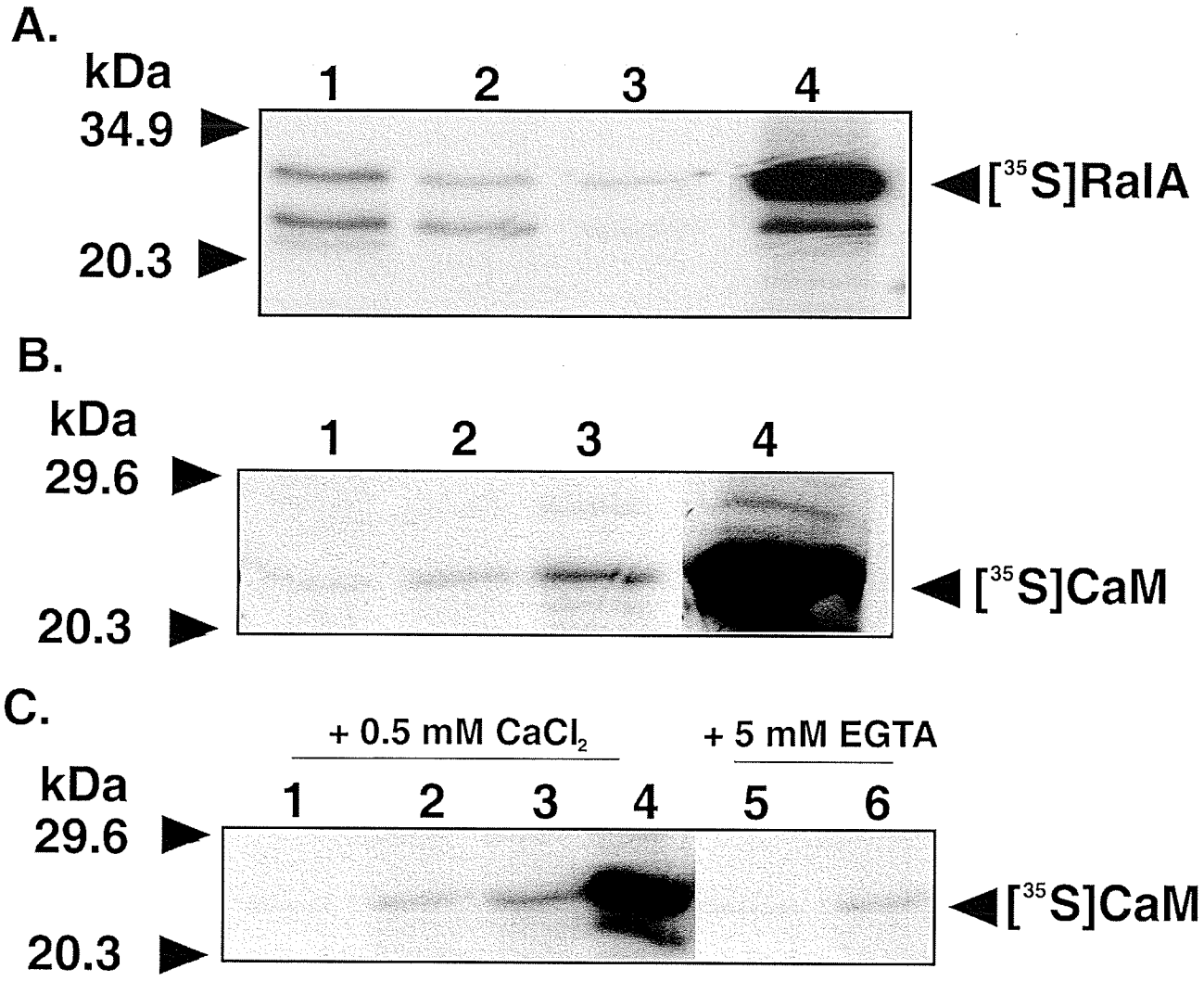

A.
kDa
1
2
3
4
34.9
20.3
[35S]RalA
B.
kDa
1
2
3
4
29.6
20.3
[35S]CaM
C.
+ 0.5 mM CaCl2
+ 5 mM EGTA
kDa
1
2
3
4
5
6
29.6
20.3
[35S]CaM

Figure 8

Fig. 8. Recombinant RalA and RalB and CaM interact specifically *in vitro* in a Ca^{2+}-dependent manner. (A) [^{35}S]Met-labelled RalA binds CaM-Sepharose *in vitro* in a Ca^{2+}-dependent manner. CaM-Sepharose beads (50 µl) were incubated with *in vitro* transcribed/translated [^{35}S]Met-labelled RalA (20% of TnT reaction mix) in an *in vitro* binding assay as described in Methods. Proteins interacting with CaM were boiled off the beads in Laemmli's sample buffer, separated by 13% SDS-PAGE, and autoradiography used to detect radiolabelled RalA that had bound to CaM-Sepharose in the presence of 0.5 mM $CaCl_2$ (Lane 1) or 5 mM EDTA (Lane 2), or control Sepharose beads in the presence of 0.5 mM $CaCl_2$ (Lane 3). *In vitro* transcribed/translated [^{35}S]RalA (10% of TnT reaction mix) was run as a control (Lane 4). (B) Sepharose-RalB and -CaM BD of RalA bind [^{35}S]CaM. Sepharose-CNBr-coupled CaM BD of RalA and recombinant RalB beads (50 µl) were incubated with *in vitro* transcribed/translated [^{35}S]CaM (20% of TnT reaction mix) in an *in vitro* binding assay as described in Methods. Autoradiography was used to detect radiolabelled CaM that had bound to blank Sepharose beads (Lane 1), Sepharose-CaM BD of RalA (Lane 2), and Sepharose-RalB (Lane 3). *In vitro* transcribed/translated [^{35}S]CaM (10% of TnT reaction mix) was run as a positive control (Lane 4). (C) The binding of Sepharose-RalB and -CaM BD of RalA to [^{35}S]CaM is Ca^{2+}-dependent. The same *in vitro* binding assay was performed with blank Sepharose beads (Lane 1), Sepharose-CaM BD of RalA (Lane 2), and Sepharose-RalB (Lane 3) beads incubated with [^{35}S]CaM in the presence of 0.5 mM $CaCl_2$, or 5 mM EGTA for GST-CaM BD of RalA (Lane 5) and GST-RalB (Lane 6). *In vitro* transcribed/translated [^{35}S]CaM (10% of TnT reaction mix) was run as a positive control (Lane 4). These experiments were repeated at least three times and gave identical results.

platelets, RalB but not RalA is present in both the particulate and cytosol fraction (Jilkina and Bhullar, 1996). Thus, cytosol was used to immunoprecipitate RalB-CaM complexes using anti-CaM antibody. RalB coprecipitated from platelet cytosol fraction derived from freshly drawn, thrombin-treated human platelets to a much greater degree with anti-CaM compared to preimmune IgG1 antibody (Fig. 9, upper panel). The extra band seen in the anti-CaM lane may be a proteolytic degradation product, protein product being translated from another start site, or background resulting from non-specific binding of the antibody. Stripping and reprobing of the membrane with anti-CaM antibody showed that CaM was present in the anti-CaM immunoprecipitate (Fig. 9, lower panel). The co-immunoprecipitation results provide further evidence that Ral and CaM interact, and that active Ral forms complexes with CaM in platelets.

5.1.4 Ral and CaM Fusion Proteins are Expressed in Transformed Yeast

Prior to performing yeast two-hybrid studies, we verified that the Ral and CaM cDNA inserts were in-frame with the GAL4 BD and AD transcription factors, respectively, and thus expressed the appropriate fusion proteins in yeast. The fusion proteins BD/cMyc/RalA (Fig. 10A, Lane 1), BD/cMyc/RalB (Fig. 10A, Lane 2), and AD/HA/CaM (Fig. 10B, Lanes 1, 2) were detected in transfected but not untransfected AH109 (Fig. 10A, Lane 3, Fig. 4B, Lane 3) and Y187 (Fig. 10B, Lane 4) yeast strains by western blotting with anti-Ral and -CaM antibodies. To reconfirm the presence of the fusion proteins, the PVDF membranes were stripped and probed with anti-c-Myc and -HA mouse monoclonal antibodies. These antibodies detected identically sized proteins (data not shown) as those detected by anti-Ral and -CaM antibodies. Results therefore

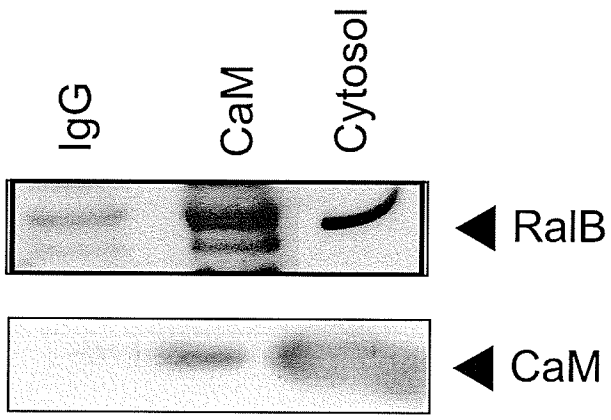


Figure 9

Fig. 9. RalB and CaM co-immunoprecipitate from human platelets. Freshly drawn human platelets were prepared as described in Methods, divided into 1 ml aliquots and treated with 0.2 U/ml thrombin for 60 seconds. Platelets were lysed by sonication, protease inhibitors added, and lysates incubated with anti-CaM monoclonal antibody or with rabbit anti-mouse IgG1. Proteins were separated by 15% SDS-PAGE and probed with anti-RalB polyclonal antibody to detect RalB coprecipitate (upper panel). Total cytosol lysate was probed as positive control. The membrane was stripped and reprobed with anti-CaM monoclonal antibody (lower panel). The experiment was repeated at least three times and gave similar results.

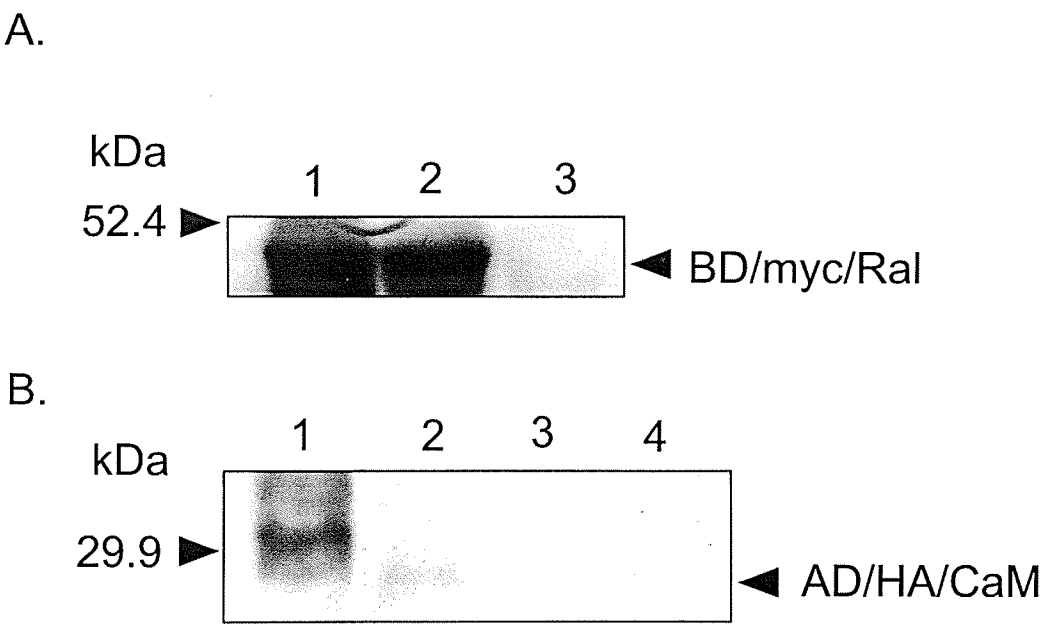


Figure 10

Fig. 10. BD/cMyc/Ral and AD/HA/CaM fusion proteins are expressed in transformed yeast. (A) RalA and RalB fusion proteins are expressed in AH109 yeast strain. Yeast strain AH109 was transfected with pGBKT7[RaIA] and pGBKT7[RalB] and all proteins subsequently expressed, including those in untransfected AH109 strain, were extracted as per Methods. The extracted proteins (10μl) were subjected to 13% SDS-PAGE, transferred to PVDF membranes, and probed with anti-RalA or -RalB antibodies to detect the presence of fusion proteins BD/cMyc/RalA (Lane 1) and BD/cMyc/RalB (Lane 2). Untransfected AH109 (Lane 3) was used as a negative control. (B) CaM fusion protein is expressed in AH109 and Y187 yeast strains. Yeast strains AH109 and Y187 were transfected with pGADT7[CaM] and all proteins subsequently expressed, including those in untransfected AH109 and Y187 strains, were extracted as per Methods. The extracted proteins (10μl) were subjected to 13% SDS-PAGE, transferred to PVDF membranes, and probed with anti-CaM antibody to detect the presence of fusion protein AD/HA/CaM in AH109 (Lane 1) and Y187 (Lane 2) strains. Untransfected AH109 (Lane 3) and Y187 (Lane 4) strains were used as negative controls.

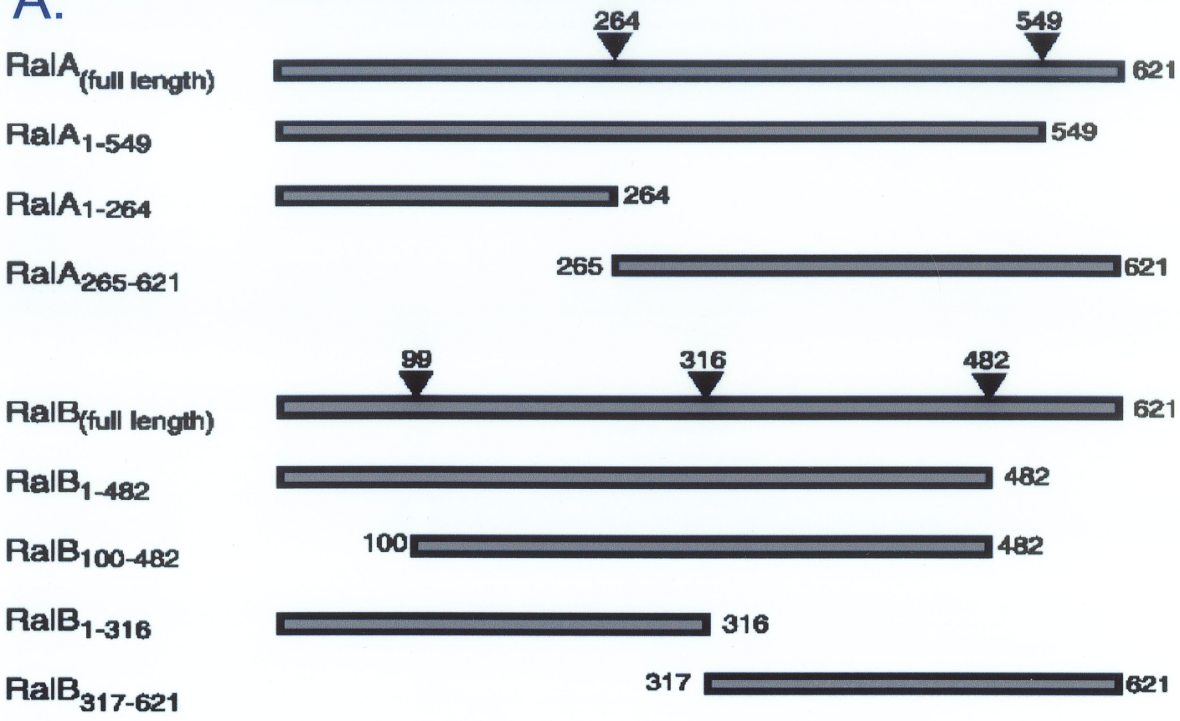
show that the yeast strains used were expressing the transfected Ral and CaM fusion proteins.

5.1.5 Calmodulin Interacts Specifically with RalA and RalB *In Vivo*

To relate *in vitro* findings to mammalian cells, it is also important to demonstrate this Ral/CaM interaction *in vivo* in a eukaryotic system. Therefore, a Y2H assay was employed, which demonstrated, for the first time, specific interaction of the Ral proteins

with CaM *in* vivo. Positive blue colonies, caused by the protein-protein-induced activation of the yeast MEL1 gene and subsequent synthesis of α-galactosidase and digestion of X-α-Gal, resulted when AH109 cells were transfected with pGADT7[CaM] and pGBKT7[RalA] or pGBKT7[RalB]. (Fig. 11B, RalA + CaM, RalB + CaM). Positive controls (i) pCL1 (Fig. 11B, pCL1), and (ii) pGBKT7[murine p53] plus pGADT7[SV40 large T antigen] (Fig. 11B, T + 53), also produced blue colonies, whereas negative controls (i) pGBKT7[lamin C] plus pGADT7[T antigen] (Fig. 11B, T + Lam), and (ii) pGBKT7[lamin C] plus pGADT7[CaM] (results not shown), and the Ral (Fig. 11B, RalA, RalB) and CaM (Fig. 11B, CaM) constructs by themselves, did not activate the MEL1 gene. This showed that the interactions between Ral and CaM were specific, and that the plasmid constructs themselves did not activate the MEL1 gene autonomously. To further show this specific interaction in yeast, AH109 cells transfected with pGBKT7[RalA] or pGBKT7[RalB] constructs were mated with Y187 yeast cells transfected with pGADT7[CaM]. Both RalA and RalB interacted with CaM to induce synthesis of α-galactosidase. The blue phenotype in both yeast transfection and mating

A.


264
549
RalA(full length)
621
RalA1-549
549
RalA1-264
264
RalA265-621
265
621
99
316
482
RalB(full length)
621
RalB1-482
482
RalB100-482
100
482
RalB1-316
316
RalB317-621
317
621

B.

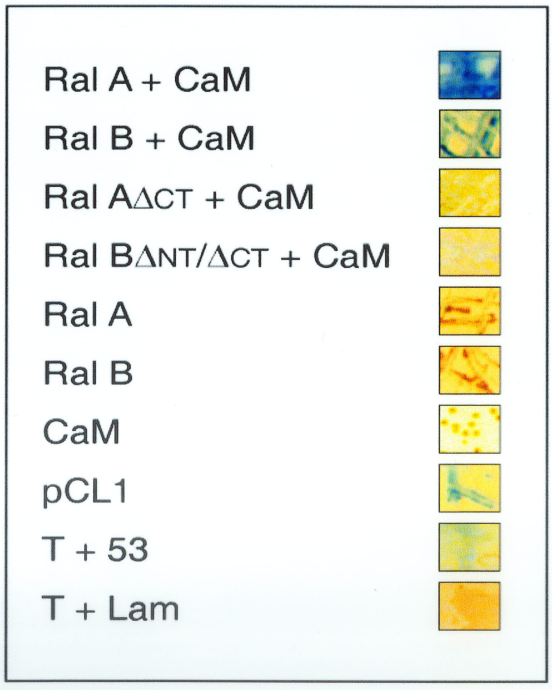

Ral A + CaM
Ral B + CaM
Ral AΔCT + CaM
Ral BΔNT/ΔCT + CaM
Ral A
Ral B
CaM
pCL1
T + 53
T + Lam

Figure 11

Fig. 11. Calmodulin interacts specifically with RalA and RalB *in vivo.* (A) Full length and truncated RalA and RalB constructs. Arrow heads denote cleavage sites, and numbers represent nucleotide base pairs. (B) Calmodulin interacts specifically with various RalA and RalB constructs *in vivo.* RalA and RalB cDNAs and their deletion constructs were subcloned into GAL4 BD expression plasmid pGBKT7, and CaM cDNA was subcloned into GAL4 AD expression plasmid pGADT7. To identify specific protein-protein interactions, yeast two-hybrid and yeast mating assays were performed as described in Methods. Colonies were monitored for blue colouration caused by the direct interaction of the GAL4 BD and AD fusion proteins. Interactions tested were pGBKT7[RalA] + pGADT7[CaM] (RalA + CaM), pGBKT7[RalB] + pGADT7[CaM] (RalB + CaM), pGBKT7[$RalA_{1-549}$] + pGADT7[CaM] ($RalA_{\Delta C-T}$ + CaM), and pGBKT7[$RalB_{100-482}$] + pGADT7[CaM] ($RalB_{\Delta N-T/\Delta C-T}$ + CaM). To ensure specificity of the systems, positive controls pCL1 (pCL1), and pGBKT7[murine p53 + pGADT7[SV40 large T-antigen] (T + 53), and negative control pGBKT7[lamin C] + pGADT7[T-antigen] (T + Lam), were also tested. AH109 yeast transformed with pGBKT7[RalA] (RalA), pGBKT7[RaIB] (RalB), or pGADT7[CaM] (CaM) were also tested for autonomous activation of the MEL1 gene. In all cases, the positive interaction phenotype persisted upon restreaking at least 3 times on high stringency drop-out medium.

experiments persisted upon restreaking 3-4 times on high stringency drop-out media. In all cases, the positive RalA/CaM colonies grew more quickly and developed a more intense blue colouration than the RalB/CaM colonies, even though western blotting showed, qualitatively, similar levels of protein were expressed by the yeast (data not shown). These results demonstrate that both RalA and RalB directly bind CaM *in vivo* in a eukaryotic system, and that RalA may bind CaM more readily than RalB.

5.1.6 Full length and C-terminally Truncated RalA and RalB Bind CaM in the Presence of Ca^{2+} *In Vitro*

The CaM BD in RalA has been shown to be at the C-terminus (Wang *et al.*, 1997) (Fig. 12). Because the two Ral proteins differ mainly at the C-terminus, we wished to determine if there was a CaM BD in another region of RalB and perhaps RalA. A CaM target data base (http:/calcium.oci.utoronto.ca/) suggests that the N-terminal region of both RalA and RalB have a propensity to form hydrophobic alpha helical wheels and that these regions have a net positive charge. These are properties required by most CaM binding domains (Fig. 12) and it is possible that RalA and RalB have N-terminal CaM BDs. We therefore tested whether removal of the C-terminal region equivalent to that proposed to contain the RalA CaM BD would eliminate CaM binding to RalA and RalB. Figure 11A shows a schematic of full length and truncated Ral constructs used in these experiments. In the yeast systems, $RalA_{1\text{-}549}$ (Fig. 11B, $RalA_{\Delta C\text{-}T}$), which has the C-terminal CaM BD deleted, $RalB_{1\text{-}482}$ (results not shown), which has the region equivalent to the C-terminal CaM BD of RalA deleted, and $RalB_{100\text{-}482}$ (Fig. 11B, $RalB_{\Delta N\text{-}T/\Delta C\text{-}T}$),

RalA MAANKPKGQNSLALHKVIMVGSGGVGKSALTLQFMYDEFVDYEPTKADSYRK

RalB MAANKSKGQSSLALHKVIMVGSGGVGKSALTLQFMYDEFVEDYEPTKADSYRK

RalA KVVLDGEEVQIDILDTAGQEDYAAIRDNYFRSGEGFLCVFSITEMESFAATADFR

RalB KVVLDGEEVQIDILDTAGQEDYAAIRDNYFRSGEGFLLVFSITEHESFTATAEFR

RalA EQILRVKEDENVPFLLVGNKSDLEDKRQVSVEEAKNRAEQWNVNYVETSAK

RalB EQILRVKAEEDKIPLLVVGNKSDLEERRQVPVEEARSKAEEWGVQYVETSAK

RalA TRANVDKVFFDLMREIRARKMEDKEKNGKKKRKSLAKRIRERCCIL

RalB TRANVDKVFFDLMREIRTKKMSENKDKNGKKSSKNKKSFKERCCLL

Fig. 12. Predicted CaM BDs in RalA and RalB. Amino acid alignments for RalA and RalB. CaM BD of RalA (green); predicted N-terminal CaM BDs in RalA and RalB (red).

which has both the putative N-terminal and C-terminal CaM BDs deleted, appeared to bind CaM, but more weakly than full length RalA and RalB. This conclusion was based solely on the subjective determination of speed of onset and degree of blue colouration of the various colonies. In support of these *in* vivo results, *in vitro* binding assays showed that Sepharose-CaM bound [^{35}S]RalA$_{1-549}$ (Fig. 13A, Lane 2) and [^{35}S]RalB$_{1-482}$ (Fig. 13B, Lane 2), as well as full length [^{35}S]RalA and [^{35}S]RalB (Fig. 13A and 13B, Lane 1, respectively) in the presence of 0.5 mM $CaCl_2$. In fact, the C-terminally truncated [^{35}S]RalB product appeared to bind Sepharose-CaM much more readily than full length [^{35}S]RalB (Fig. 13B, compare Lanes 1 and 2). However, Sepharose-CaM did not significantly bind more of the doubly truncated RalB product ([^{35}S]RalB$_{100-482}$) (Fig. 13B, Lane3) over that of control Sepharose-CNBr beads (Fig. 13B, Lane 6). Results with RalB$_{1-482}$ showed that an equal or greater amount of [^{35}S]RalB$_{1-482}$ bound CaM-Sepharose in the presence of EDTA (Fig. 13C, Lane 2) as in the presence of $CaCl_2$ (Fig. 13C, Lane 1). As shown above (Fig. 8), the CaM/full-length Ral interaction was Ca^{2+}-dependent because 5 mM EGTA markedly decreased the binding detected in the presence of 0.5 mM $CaCl_2$. These results suggest there is more than one CaM BD in RalA, and probably RalB, and that RalA and RalB have an N-terminal and a C-terminal CaM BD. It is also possible that RalB$_{1-482}$ binds CaM more strongly in the absence of Ca^{2+}.

5.1.7 RalA and RalB Have an N-terminal Ca^{2+}-Independent and a C-terminal Ca^{2+}-Dependent CaM BD

To further confirm the presence of more than one CaM BD in Ral, and to test their Ca^{2+}-dependence, [^{35}S]Met-labelled N- and C-terminal halves of RalA and RalB were

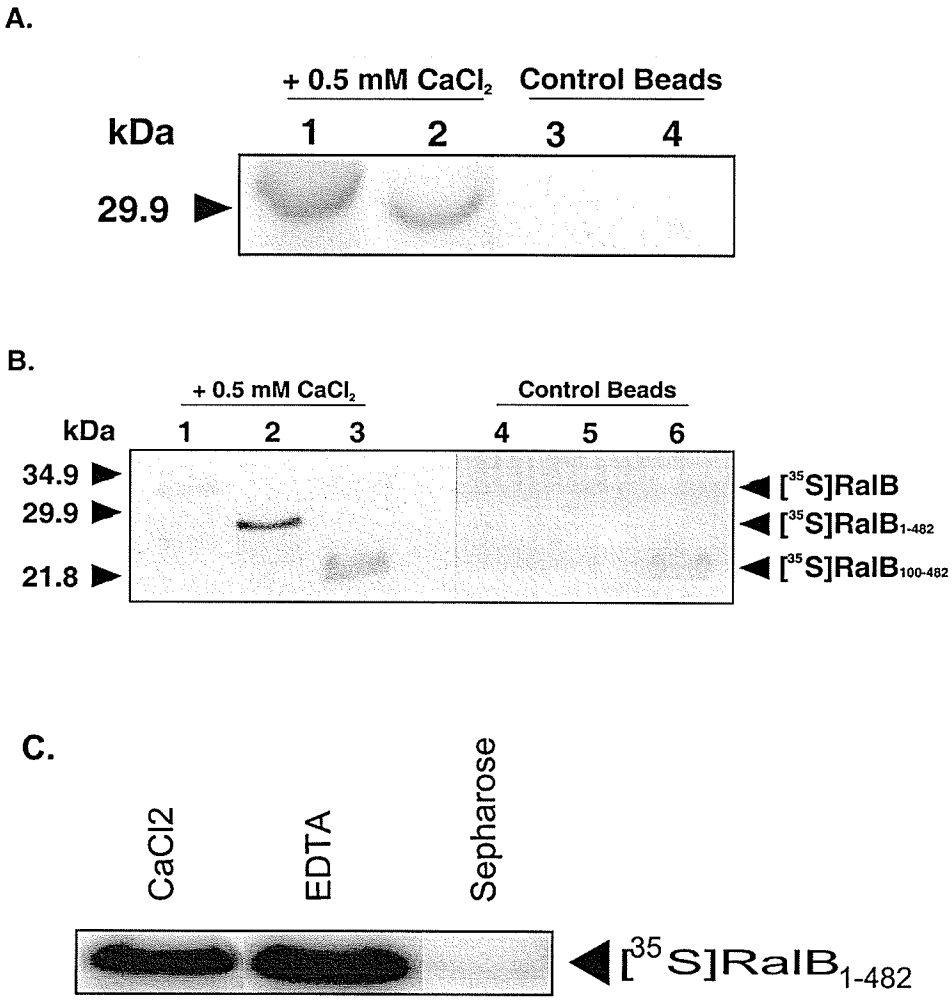

A.
+ 0.5 mM CaCl2
Control Beads
kDa
1
2
3
4
29.9
B.
+ 0.5 mM CaCl2
Control Beads
kDa
1
2
3
4
5
6
34.9
29.9
21.8
[35S]RalB
[35S]RalB1-482
[35S]RalB100-482
C.
CaCl2
EDTA
Sepharose
[35S]RalB1-482

Figure 13

Fig. 13. Full length and C-terminally truncated RalA and RalB bind CaM in the presence of Ca^{2+} *in vitro*. (A) RalA and $RalA_{1-549}$ bind CaM *in vitro* in the presence of Ca^{2+}. In an *in vitro* binding assay described in Methods, 20% of *in vitro*-transcribed/translated [^{35}S]Met-labelled RalA (Lane 1) and $RalA_{1-549}$ (Lane 2) were incubated with 50 µl CaM-Sepharose 4B beads in the presence of 0.5 mM $CaCl_2$, or incubated with 50 µl control Sepharose 4B beads plus 0.5 mM $CaCl_2$ as a negative control (Lanes 3, 4 respectively). (B) C-terminally truncated RalB binds CaM more readily than full length RalB *in vitro*. In an *in vitro* binding assay described in Methods, 20% of *in vitro* translated [^{35}S]RalB (Lane 1), [^{35}S]$RalB_{1-482}$ (Lane 2), and [^{35}S]$RalB_{100-482}$ (Lane 3) were incubated with 50 µl CaM-Sepharose 4B beads in the presence of 0.5 mM $CaCl_2$, or with 50 µl control Sepharose 4B beads plus 0.5 mM $CaCl_2$ as a negative control (Lanes 4, 5, 6 respectively). (C) C-terminally truncated RalB binds CaM in a Ca^{2+}-independent manner. In an *in vitro* binding assay described in Methods, 20% of *in vitro* translated [^{35}S]$RalB_{1-482}$ was incubated with 50 µl CaM-Sepharose 4B beads in the presence of 0.5 mM $CaCl_2$ (Lane 1) or 5 mM EDTA (Lane 2), or with 50 µl control Sepharose 4B beads plus 0.5 mM $CaCl_2$ as a negative control (Lane 3). In A-C, bound proteins were recovered from beads by boiling in 1 × Laemmli's buffer and separated by 13% SDS-PAGE. The gel was vacuum-dried and autoradiography used to visualize radioactivity associated with the proteins. These experiments were repeated at least three times and gave identical results.

tested for interaction with CaM-Sepharose. Initially, the pGBKT7[$RalA_{1-264}$], pGBKT7[$RalA_{265-621}$], pGBKT7[$RalB_{1-316}$] and pGBKT7[$RalB_{317-621}$] constructs were subject to *in vitro* transcription/translation in the presence of [^{35}S]Met to ensure the Ral cDNAs were in frame with the cMyc epitope tags and could thus produce appropriate fusion proteins. Autoradiography showed that [^{35}S]Met-labelled cMyc/$RalA_{1-264}$ (Fig.14, upper panel, Lane 7) cMyc/$RalA_{265-621}$ (Fig. 14, upper panel, Lane 8), cMyc/$RalB_{1-316}$ (Fig. 14, lower panel, Lane 7) and cMyc/$RalB_{317-621}$ (Fig. 14, lower panel, Lane 8) fusion proteins were translated. [^{35}S]Met-labelled $RalA_{1-264}$ (Fig. 14, upper panel, Lanes 1, 3, 5, 7) and $RalB_{1-316}$ (Fig. 14, lower panel, Lanes 1, 3, 5, 7), both containing the approximate N-terminal half of each protein, strongly bound CaM-Sepharose in the presence of either 0.5 mM $CaCl_2$ (Fig. 14, Lanes 1) or 5 mM EDTA (Fig. 14, Lanes 3), whereas [^{35}S]Met-labelled $RalA_{265-621}$ (Fig. 14, upper panel, Lanes 2, 4, 6, 8) and $RalB_{317-621}$ (Fig. 14, lower panel, Lanes 2, 4, 6, 8), both containing the approximate C-terminal half of each protein, strongly bound CaM-Sepharose in the presence of Ca^{2+} (Fig. 14, lanes 2) but significantly less or not at all in the presence of EDTA (Fig. 14, lanes 4). The reaction was specific because neither Rals bound to the control Sepharose 4B beads (Fig. 14, lanes 5, 6). These results suggest that the C-terminal halves of RalA and RalB contain a Ca^{2+}-dependent CaM BD motif, whereas the N-terminal halves contain a Ca^{2+}-independent CaM BD. It is also possible, comparing lanes 1 and 3 (Fig. 14, upper and lower panel), that Ca^{2+} may partially inhibit CaM binding to the N-terminal CaM BD. Results showing [^{35}S]Met-labelled $RalB_{1-482}$ binding CaM-Sepharose in a Ca^{2+}-independent manner (Fig. 13C) indicate that the last 40 or so amino acids that partially inhibit, in some way, the RalB/CaM interaction *in vitro*, also contain the Ca^{2+}-dependent CaM BD. Although

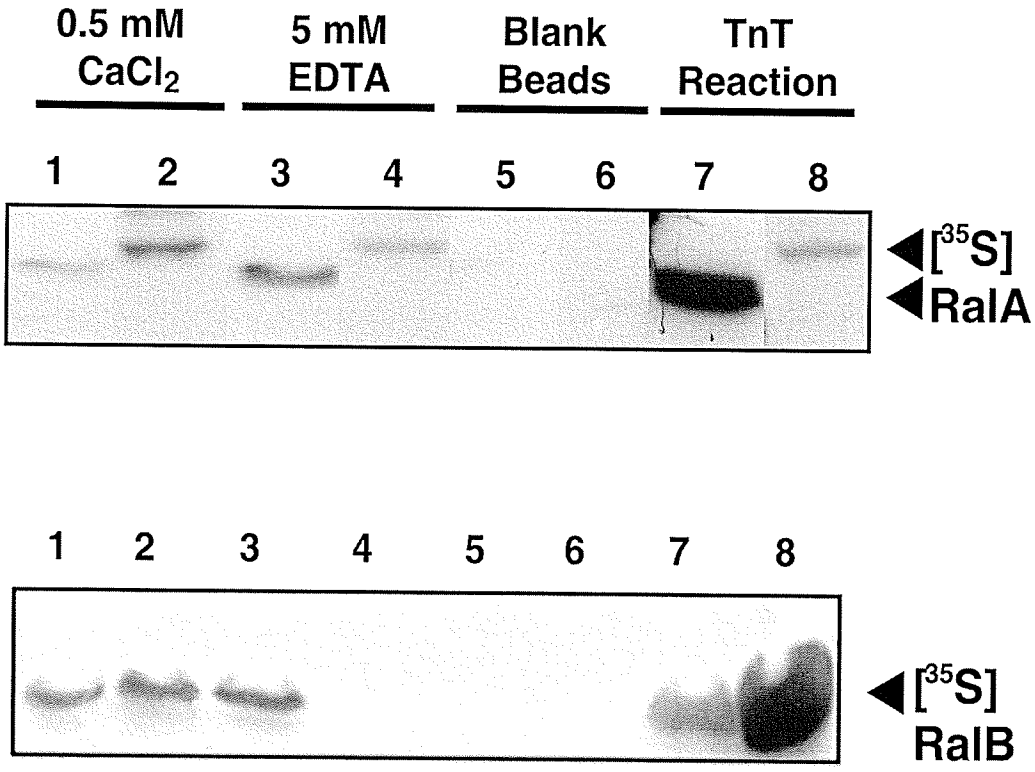

0.5 mM CaCl2
5 mM EDTA
Blank Beads
TnT Reaction
1 2 3 4 5 6 7 8
[35S] RalA
1 2 3 4 5 6 7 8
[35S] RalB

Figure 14

Fig. 14. RalA and RalB contain an N-terminal Ca^{2+}-independent and a C-terminal Ca^{2+}-dependent CaM BD. In an *in vitro* binding assay described in Methods, 20% of *in vitro*-transcribed-translated [^{35}S]Met-labelled $RalA_{1-264}$ (upper panel, Lanes 1, 3, 5, 7), $RalA_{265-621}$ (upper panel, Lanes 2, 4, 6, 8), $RalB_{1-316}$ (lower panel, lanes 1, 3, 5, 7), and $RalB_{317-621}$ (lower panel, Lanes 2, 4, 6, 8) were incubated with 50 µl CaM-Sepharose 4B beads in the presence of 0.5 mM $CaCl_2$ or 5 mM EDTA as indicated, or incubated with 50 µl control Sepharose 4B beads plus 0.5 mM $CaCl_2$ as a negative control (Blank beads). *In vitro* transcribed/translated [^{35}S]Met-labelled Ral constructs (TnT reaction) were run as positive controls. Bound proteins were recovered from beads by boiling in 1 × Laemmli's buffer and separated by 15% SDS-PAGE. The gel was vacuum-dried and autoradiography used to visualize radioactivity associated with the proteins. These experiments were repeated at least three times and gave identical results.

removal of large regions of the Ral proteins may have altered the normal 3-D structure of the remaining portions and therefore changed their CaM binding properties, the results do suggest that RalA and RalB have two CaM interacting regions that vary in their Ca^{2+} dependence.

5.1.8 CaM is Required for the Thrombin-Induced Activation of RalA and RalB in Human Platelets

We next wanted to examine the molecular mechanisms involved in Ral activation. First, we used GST-RRBD to pull down activated Ral to determine if CaM plays a role in the activation of Rals. The Ral BD motif of RIP1 selectively binds GTP-bound, but not GDP-bound Ral (Wolthuis *et al.*, 1998b). Initially, it was assessed whether GST-RRBD was expressed in AD202 cells made competent by the rubidium chloride method (Promega). Bacterial cultures transfected with pGEX4T2[RRBD] were stimulated with IPTG to express GST-RRBD The proteins were separated by 13% SDS-PAGE and the gel stained with Coomassie blue. A distinct band of expected molecular mass was detected (Fig. 15A). To check specificity of our GST-RRBD construct, 1 ml thrombin-stimulated and unstimulated freshly drawn human platelets were subject to GST-RRBD and control GST pull-down experiments, as described in Methods (Fig. 15B). Only thrombin-stimulated platelets showed significant amounts of RalA-GTP (Fig. 15B, upper panel) and RalB-GTP (Fig. 15B, lower panel), with unstimulated platelets showing negligible amounts (Fig. 15B). The reaction was specific because GST control beads did not bind activated Ral in either unstimulated or thrombin-stimulated platelets (Fig. 15B). These results confirm that our RRBD construct binds only active Ral.

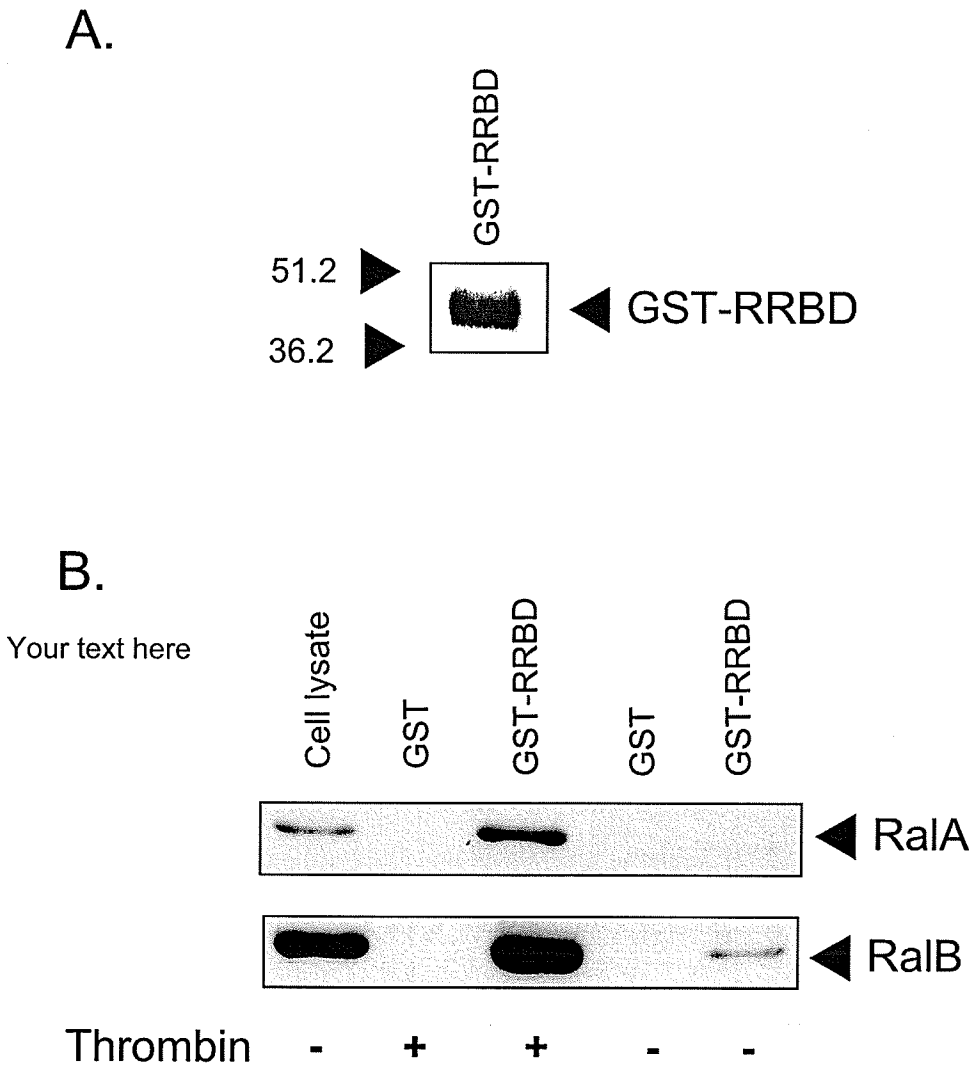

A.
GST-RRBD
51.2
36.2
GST-RRBD
B.
Your text here
Cell lysate
GST
GST-RRBD
GST
GST-RRBD
RalA
RalB
Thrombin - + + - -

Figure 15

Fig. 15. GST-RRBD specifically binds activated Ral in human platelets. (A) GST-RRBD is expressed in AD202 cells. AD202 cells were transfected with pGEX-4T-2[RRBD] and proteins stimulated to be expressed by IPTG as described in Methods. Proteins from bacterial lysates were separated by 13% SDS-PAGE and stained with Coomassie blue. (B) GST-RRBD binds active but not inactive Ral in human platelets. Freshly drawn human platelets were treated for 75 seconds with 0.2 U/ml thrombin. After thrombin treatments, the platelets were lysed in 3 x Ral binding buffer and incubated with 60 μl GST-RRBD precoupled to GSH-agarose beads to recover GTP-bound Ral. Beads were washed 4 times in Ral binding buffer, proteins were boiled off in Laemmli buffer, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA (upper panel) or –RalB (lower panel) antibodies. These experiments were repeated at least three times and gave identical results.

The next step was to determine if CaM was involved in the activation of Ral in platelets. Thrombin led to full activation of RalA (Fig. 16, upper panel) and RalB (Fig. 16, lower panel) within 10 seconds. This activation was eliminated in the presence of the CaM inhibitor W7 but not W5. W5 was used at a low non-CaM-inhibiting concentration to show that CaM inhibitors do not autonomously prevent Ral activation. These results suggest that CaM functions in the activation and regulation of RalA and RalB in platelets.

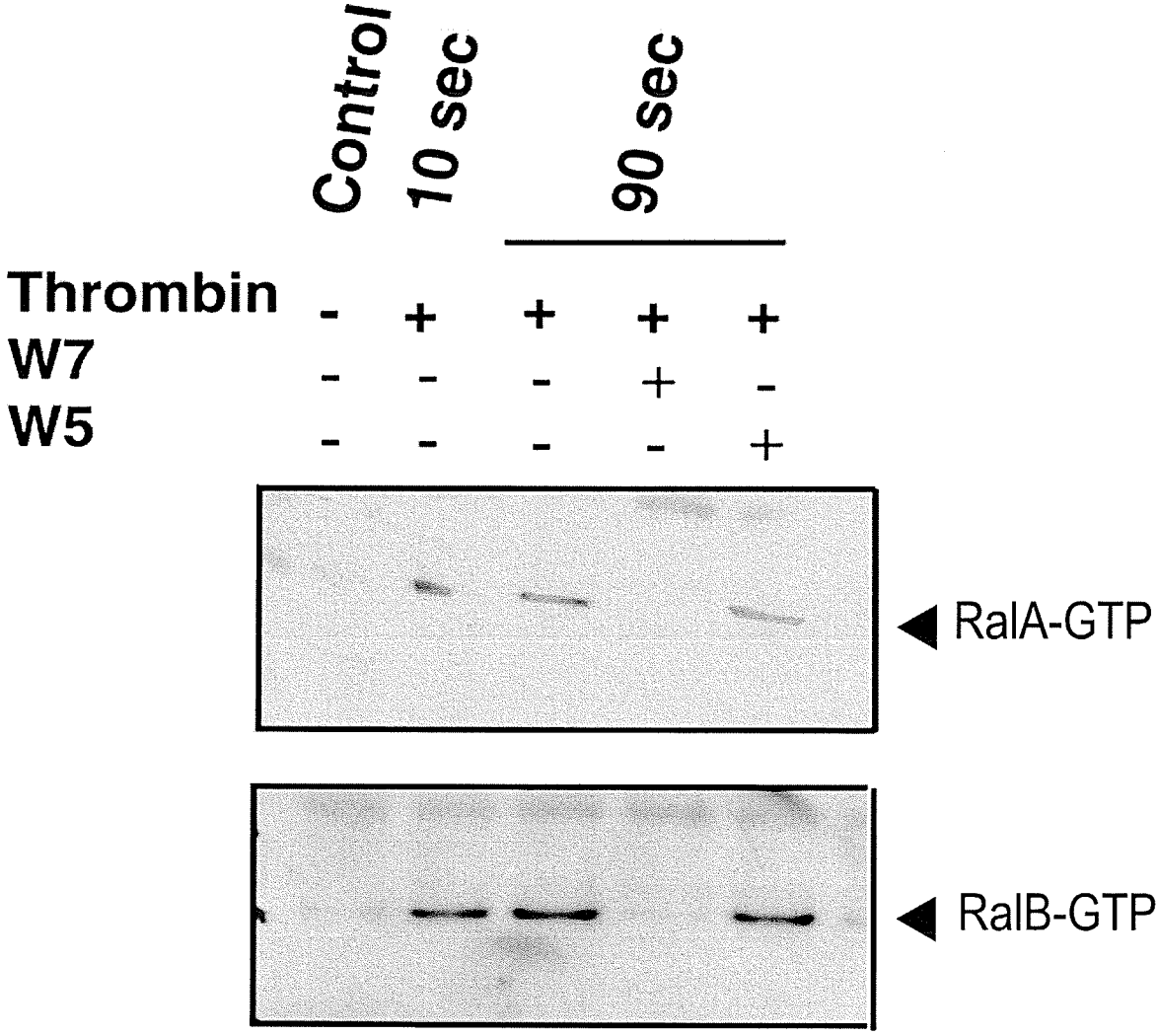


Figure 16

Fig. 16. Calmodulin is required for the thrombin-induced activation of RalA and RalB in human platelets. Freshly drawn human platelets were prepared as described in Methods, divided into 500 μl aliquots, and incubated for 30 minutes at 37°C prior to thrombin treatment (0.2 U/ml). Ten minutes before thrombin treatment, indicated aliquots were treated with either 50 μM W7 or W5. After thrombin treatments for 10 or 90 seconds, the platelets were lysed in 3 x Ral buffer and incubated with 60 μl GST-RRBD precoupled to GSH-agarose beads to recover GTP-bound Ral. Beads were washed 4 times in Ral buffer, proteins were boiled off in Laemmli buffer, and collected Ral-GTP was identified by western analysis with anti-RalA monoclonal (upper panel) or anti-RalB polyclonal (lower panel) antibody. These experiments were repeated at least three times and gave identical results.

5.2 Regulation of Ral Activation in Mammalian Cells

5.2.1 Calmodulin is Required for the EGF-Induced Activation of Ral in A7r5 and MCF7 Cells but not in HeLa Cells

Because platelets are non-proliferating cells, we wished to examine mechanisms of Ral activation in proliferating cells. To this end, and because Ral is a major down-stream effector of EGF stimulation, we studied activation of Ral in response to EGF treatment in mammalian cell lines. As described above, Ral is a CaM binding protein and can require Ca^{2+} and CaM for activation (Clough *et al.*, 2002). Therefore, to determine whether CaM is required for the EGF-induced Ras/RalGEF-dependent activation of Ral, quiescent A7r5, HeLa and MCF7 cells (Fig. 17) were treated with EGF in the presence or absence of the CaM inhibitor W7, and cell lysates incubated with GST-RRBD coupled to GSH-agarose beads to pull down activated Ral. EGF activated RalA (Fig. 17A) and RalB (results not shown) in A7r5 cells, and RalA and RalB in MCF7 cells (Fig. 17B) above basal levels. W7 inhibited the EGF-induced activation of RalA (Fig. 17A; 50% +/- 18% SD) and RalB (results not shown) in A7r5 cells, as well as in MCF7 (Fig. 17B, 77.5% +/- 3% SD and 67% +/- 6.5% SD, respectively) cells. However, in HeLa cells (Fig. 17C), quantification showed that EGF-induced activation of RalA and RalB was unaffected by W7, even though we have established that Ral binds CaM in these cells (results not shown). Higher concentrations of W7 did not cause any further inhibition of RalA and RalB in the three cell types (results not shown). The results demonstrate that EGF-induced Ral activation in A7r5 and MCF7 cells is CaM-dependent, and is CaM-independent in HeLa cells.

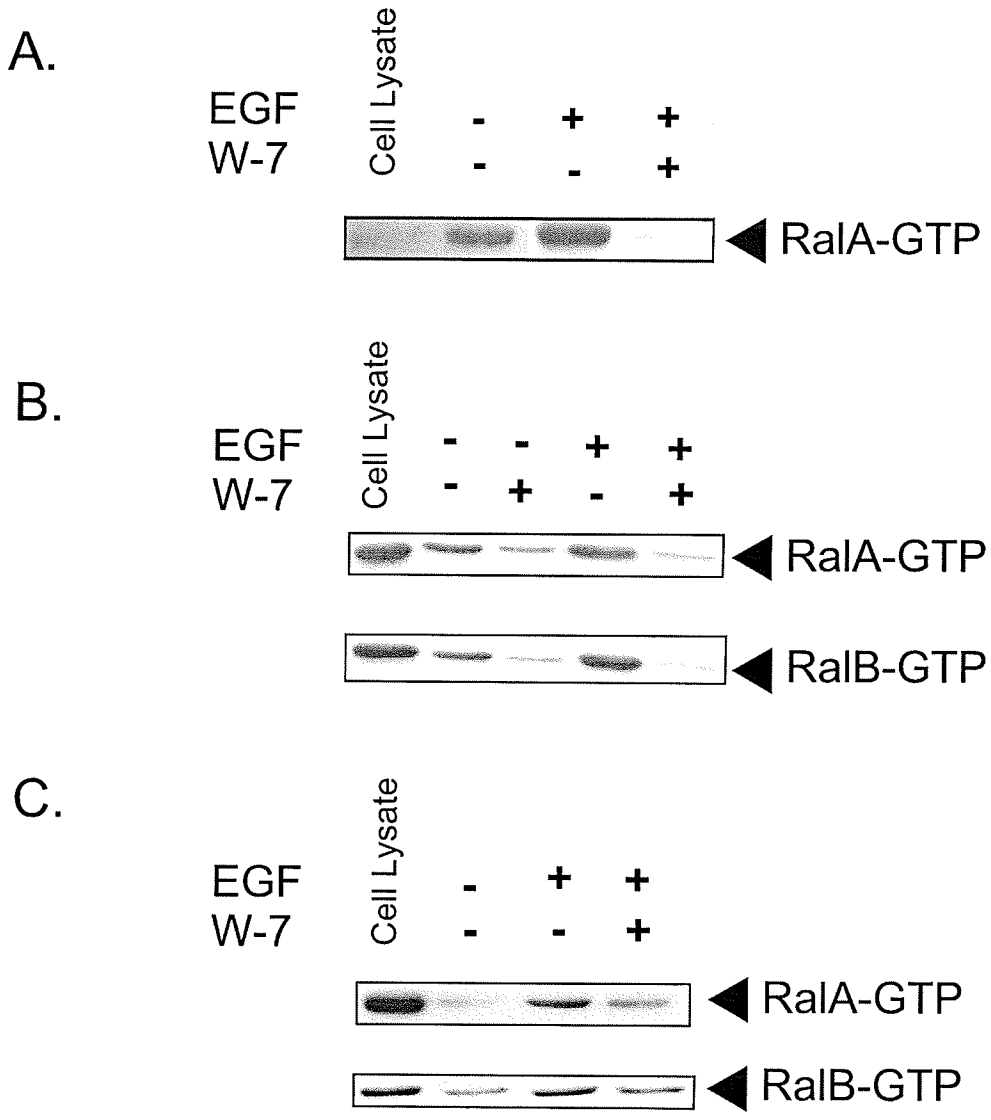

A.
EGF
W-7
Cell Lysate
- + +
- - +
RalA-GTP
B.
EGF
W-7
Cell Lysate
- - + +
- + - +
RalA-GTP
RalB-GTP
C.
EGF
W-7
Cell Lysate
- + +
- - +
RalA-GTP
RalB-GTP

Figure 17

Fig. 17. Calmodulin is required for the EGF-induced activation of Ral in A7r5 and MCF7 cells but not in HeLa cells. Quiescent, subconfluent A7r5 (A), MCF7 (B) and HeLa (C) cells were treated for 30 minutes with 50 μM W7 prior to a 15 minute treatment with 10 ng/ml recombinant EGF. As described in Methods, activated Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA or –RalB antibodies. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown). The experiments were repeated at least three times and gave identical results.

5.2.2 Ca^{2+} is Required for the Basal and EGF-induced Activation of Ral in A7r5 and HeLa Cells

Ral is activated by Ca^{2+}, and the EGFR may be involved in Ca^{2+}-dependent signalling pathways. This is because early events induced by EGF result in increased intracellular Ca^{2+} concentration and activation of PLCγ (Schlessinger, 2000; Prenzel *et al.*, 2001). To determine whether Ca^{2+} is required for the EGF-induced activation of Ral, A7r5 (Fig. 18A) and HeLa (Fig. 18B) cells were treated with EGF in the presence or absence of the Ca^{2+} chelator 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (BAPTA-AM), and activated Ral pulled down with GST-RRBD coupled to GSH-agarose beads. The EGF-induced activation of RalA in A7r5 cells (Fig. 18A), and of RalA (Fig. 18B, upper panel) and RalB (Fig. 18B, lower panel) in HeLa cells was decreased by BAPTA-AM. Higher concentrations of BAPTA-AM did not cause any further inhibition of RalA and RalB (data not shown). These results indicate that Ca^{2+} is required for the EGF-induced activation of RalA and RalB in A7r5 and HeLa cells.

5.2.3 PLC is Required for the Basal and EGF-induced Activation of Ral in A7r5 cells, and of RalB but not RalA in MCF7 and HeLa Cells

Because phospholipase Cγ (PLCγ) binds to the EGF-stimulated EGFR (Schlessinger, 2000; Prenzel *et al.*, 2001), we speculate that one source of the Ral-activating Ca^{2+} is the EGF-induced rise in intracellular Ca^{2+} that occurs through the PLCγ/phosphoinositide signaling pathway (Schlessinger, 2000; Prenzel *et al.*, 2001). Therefore, to determine if PLC is required for the EGF-induced activation of Ral,

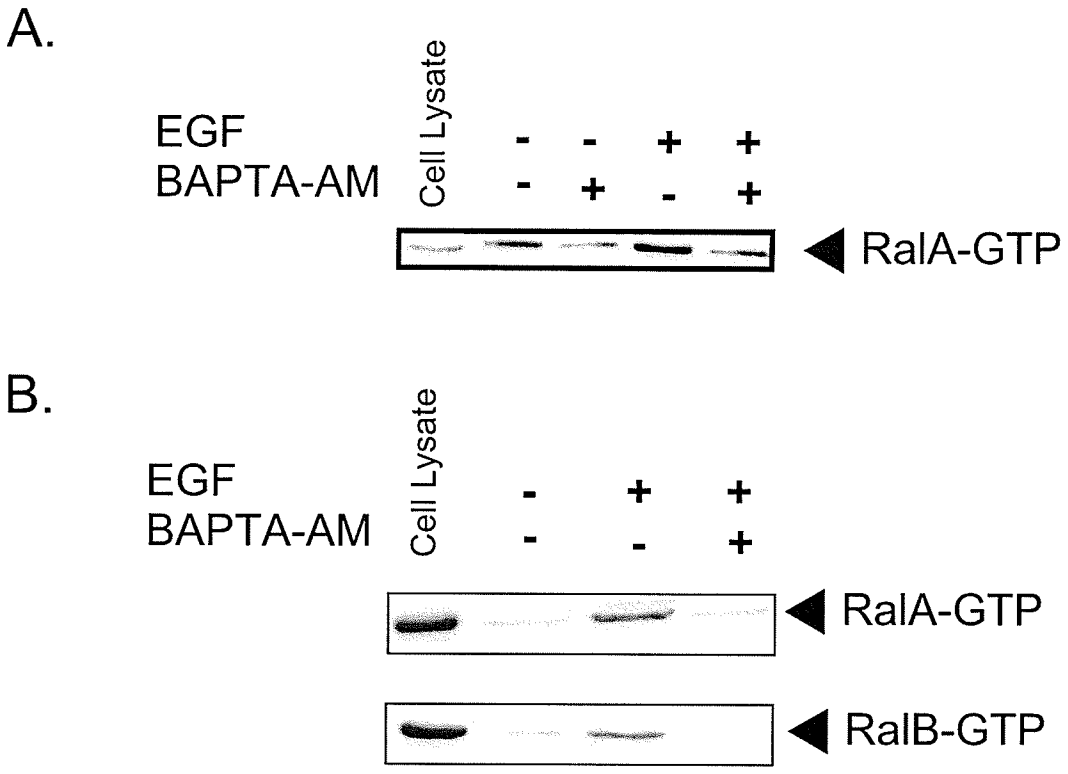

A.
EGF
BAPTA-AM
Cell Lysate
- - + +
- + - +
RalA-GTP
B.
EGF
BAPTA-AM
Cell Lysate
- + +
- - +
RalA-GTP
RalB-GTP

Figure 18

Fig. 18. Calcium is required for the EGF-induced activation of Ral in A7r5 and HeLa cells. Quiescent, subconfluent A7r5 (A) and HeLa (B) cells were treated for 30 minutes with 50 μM BAPTA-AM prior to a 15 minute treatment with 10 ng/ml recombinant EGF. As described in Methods, activated Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA or –RalB antibodies. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown). The experiments were repeated at least three times and gave identical results.

quiescent A7r5, MCF7 and HeLa cells were stimulated with EGF in the presence or absence of the PLC inhibitor U73122, and activated Ral pulled down by GST-RRBD coupled to GSH-agarose beads (Fig. 19). U73122 inhibited the EGF-induced activation of RalA in A7r5 cells (Fig. 19A; 37% +/- 16% SD). In MCF7 and HeLa cells, quantification demonstrated that U73122 did not inhibit the EGF-induced activation of RalA (19B and C, upper panel). However, U73122 did inhibit the activation of RalB in both MCF7 (Fig. 19B, lower panel; 42% +/- 2% SD) and HeLa (Fig. 19C, lower panel; 65% +/- 4% SD) cells. Higher concentrations of U73122 did not cause any further inhibition of RalA and RalB. Therefore, the EGF-induced activation of Ral in A7r5 cells, and RalB in MCF7 and HeLa cells, occurs via a PLC-dependent pathway, whereas RalA activation in MCF7 and HeLa cells does not require PLC. These findings suggests that PLC is activated by EGF, that the PLC/phosphoinositide pathway is required to a variable extent, depending on cell type, for basal and EGF-induced Ral activation, and that RalA and RalB may be differentially regulated in MCF7 and HeLa cells.

5.2.4 PKCδ is Involved in the EGF-induced Activation of Ral in A7r5 Cells, but not in HeLa Cells

Because PLC is variably involved in Ral activation, we wished to determine whether the PLC-induced production of DAG and subsequent activation of PKC is involved in EGF-induced activation of Ral. Therefore, A7r5 and HeLa cells were treated with EGF in the presence or absence of Go6976 (an inhibitor of PKCα, β, and γ) or Rottlerin (an inhibitor of PKCδ), and activated Ral pulled down by GST-RRBD coupled to GSH-agarose beads (Fig. 20). We had previously determined that Ro318220 (a general

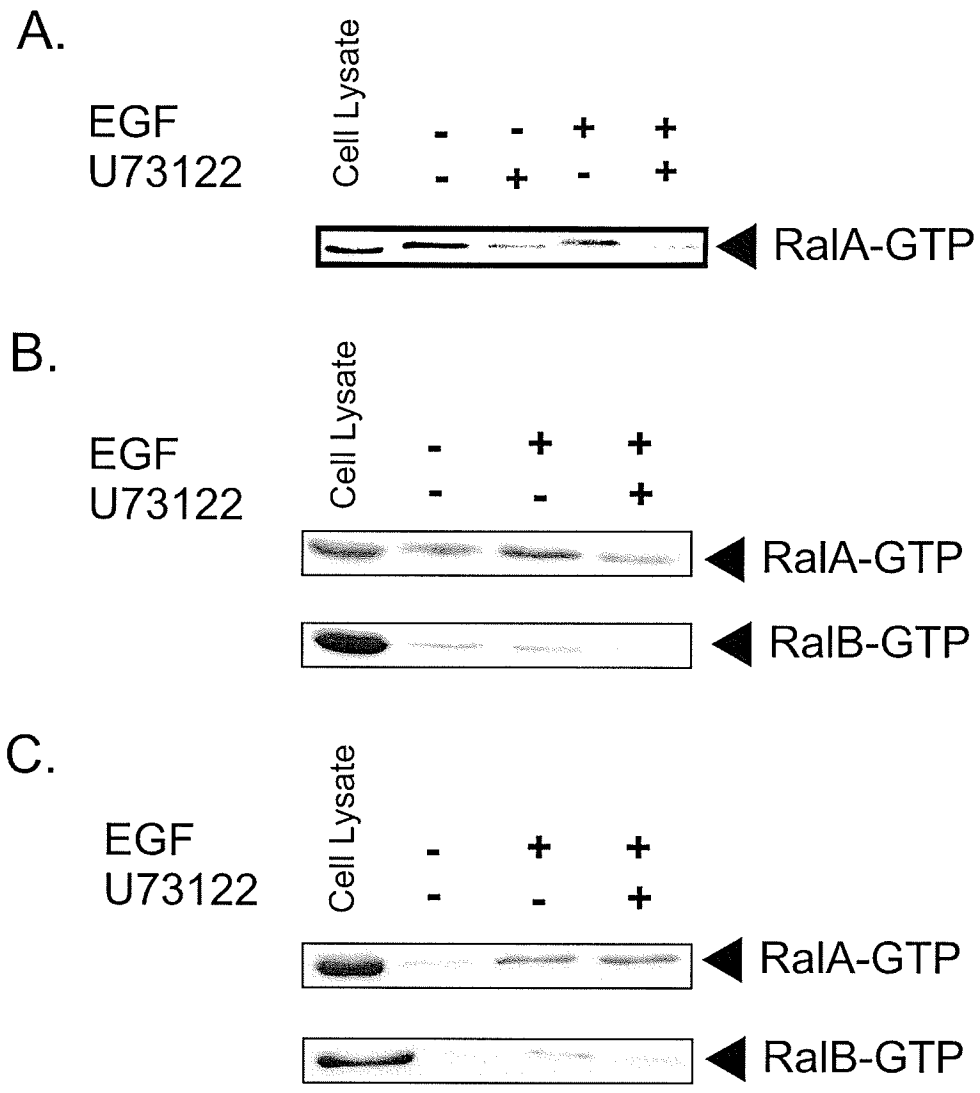

A.
EGF
U73122
Cell Lysate
- - + +
- + - +
RalA-GTP
B.
EGF
U73122
Cell Lysate
- + +
- - +
RalA-GTP
RalB-GTP
C.
EGF
U73122
Cell Lysate
- + +
- - +
RalA-GTP
RalB-GTP

Figure 19

Fig. 19. Phospholipase C is required for the EGF-induced activation of Ral in A7r5 cells, and of RalB but not RalA in MCF7 and HeLa cells. Quiescent, subconfluent A7r5 (A), MCF7 (B) and HeLa (C) cells were treated for 5 minutes with 10 μM U73122 prior to a 15 minute treatment with 10 ng/ml recombinant EGF. As described in Methods, activated Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA or –RalB antibodies. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown. The experiments were repeated at least three times and gave identical results.

Fig. 20. Protein kinase Cδ is required for the EGF-induced activation of Ral in A7r5 but not HeLa cells. Quiescent, subconfluent A7r5 (A) and HeLa (B, C) cells were treated for 30 minutes with 10 nM Go6976 or 10 μM Rottlerin prior to a 15 minute treatment with 10 ng/ml recombinant EGF. As described in Methods, activated Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA or –RalB antibodies. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown). The experiments were repeated at least three times and gave identical results.

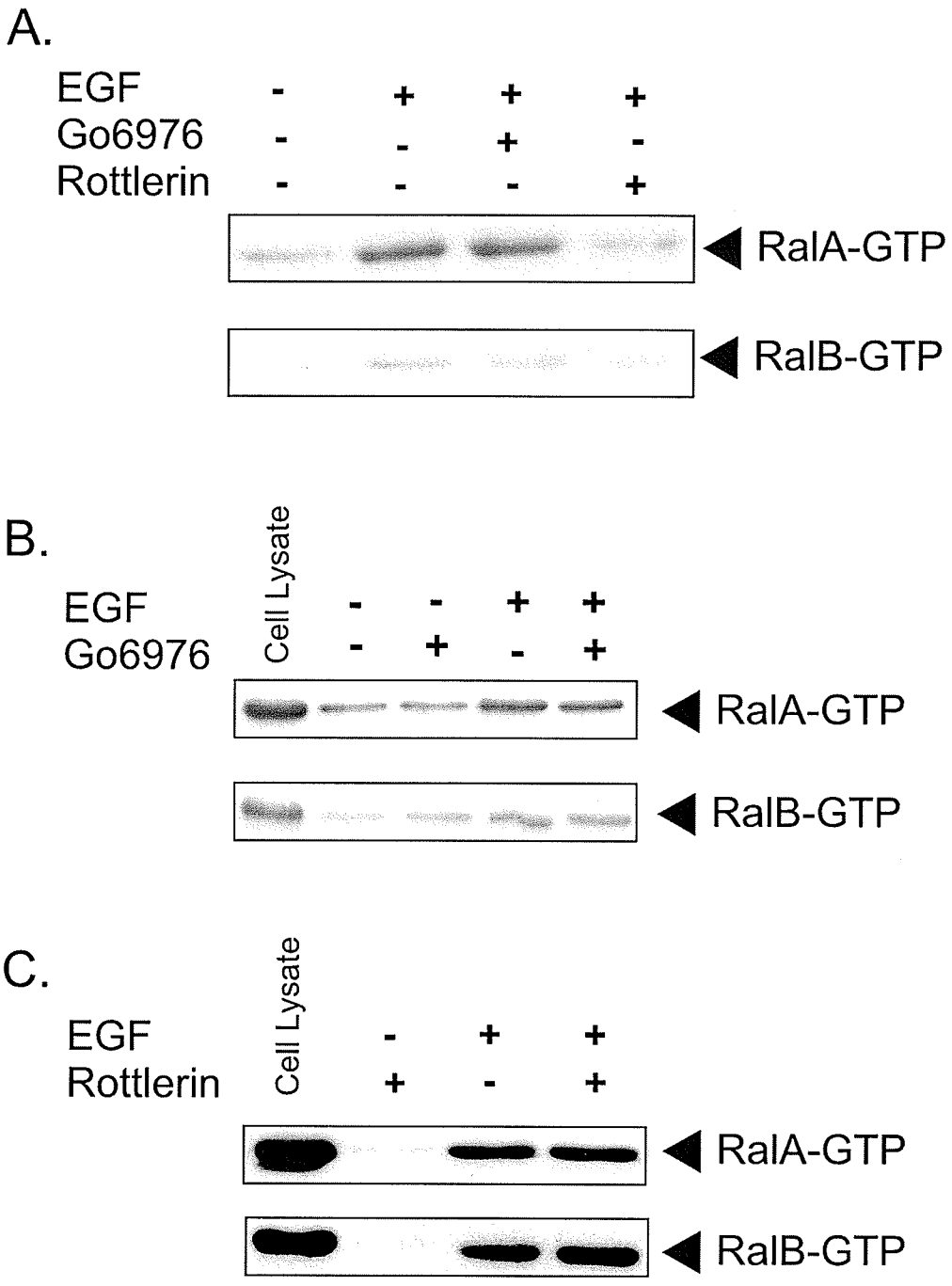

A.
EGF - + + +
Go6976 - - + -
Rottlerin - - - +
RalA-GTP
RalB-GTP
B.
Cell Lysate
EGF - - + +
Go6976 - + - +
RalA-GTP
RalB-GTP
C.
Cell Lysate
EGF - + +
Rottlerin + - +
RalA-GTP
RalB-GTP

Figure 20

PKC inhibitor), inhibits EGF-induced Ral activation in A7r5 cells. In A7r5 cells none of the inhibitors affected basal Ral activation (results not shown). However, Rottlerin (Fig. 20A) inhibited EGF-induced RalA and RalB activation. Pre-treatment of HeLa cells with Go6976 (Fig. 20B) or Rottlerin (Fig. 20C) did not suppress or enhance EGF-induced RalA (Fig. 20B and C, upper panel) or RalB (Fig. 20B and C, lower panel) activation. Go6976 (Fig, 20B) and Rottlerin (results not shown) alone had no effect on basal Ral activation. Higher concentrations of inhibitors did not cause any further inhibition of RalA and RalB. These results suggest that EGF-induced Ral activation in A7r5, but not HeLa cells, is PKCδ-dependent.

5.2.5 PMA Induces Activation of Ral in A7r5 and HeLa Cells via PKCδ in a Ca^{2+}/CaM-dependent Manner

To determine whether direct activation of PKC activates Ral, quiescent A7r5 and HeLa cells were treated with PMA, and activated Ral pulled down by GST-RRBD coupled to GSH-agarose beads. In both A7r5 (Fig. 21A) and HeLa (Fig. 21B) cells, PMA activated RalA and RalB in a time-dependent manner over 30 minutes. This PMA-dependent RalA (Fig. 22A and B, upper panels) and RalB (Fig. 22A and B, lower panels) activation was subsequently determined to be inhibited by W7 (Fig. 22, upper panel) and BAPTA-AM (Fig. 22, lower panel) in A7r5 cells. In addition, Rottlerin inhibited PMA-dependent RalA (Fig. 23A and B, upper panels) and RalB (Fig. 23A and B, lower panels) activation in A7r5 (Fig. 23A) and HeLa (Fig. 23B; 53% +/- 7.5% SD and 53% +/- 5% SD, respectively) cells. These results suggest that the direct activation of PKC by PMA

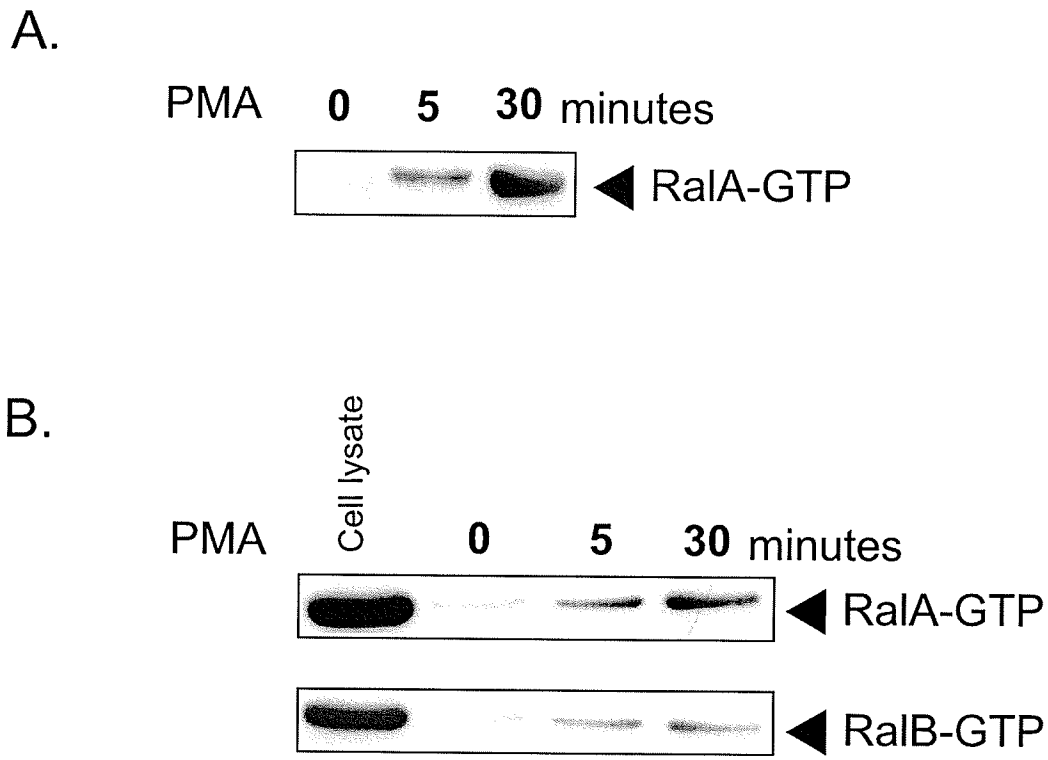

A.
PMA
0
5
30 minutes
RalA-GTP
B.
Cell lysate
PMA
0
5
30 minutes
RalA-GTP
RalB-GTP

Figure 21

Fig. 21. PMA induces activation of Ral in A7r5 and HeLa cells in a time-dependent manner. Quiescent, subconfluent A7r5 (A) and HeLa (B) cells were treated for 5 and 30 minutes with 1 μM PMA. As described in Methods, activated Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA (A, B) or –RalB (B) antibodies. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown). The experiments were repeated at least three times and gave identical results.

Fig. 22. PMA-induced activation of Ral occurs in a Ca^{2+}/CaM-dependent manner. Quiescent, subconfluent A7r5 cells were treated with 50 μM W7 (A) or 50 μM BAPTA-AM (B) 30 minutes prior to a 5 minute treatment with 1 μM PMA, and active Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA (A and B, upper panels) or –RalB (A and B, lower panels) antibodies. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown). The experiments were repeated at least three times and gave identical results.

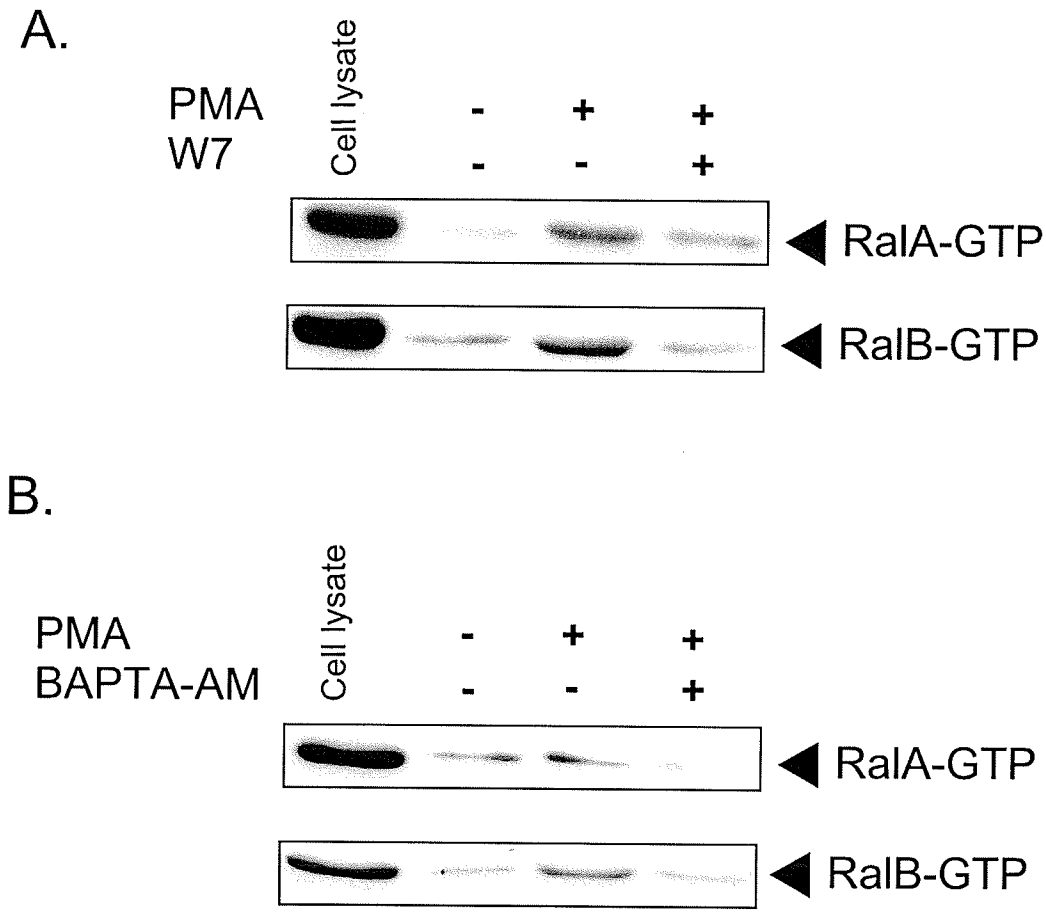

A.
Cell lysate
PMA
W7
- + +
- - +
RalA-GTP
RalB-GTP
B.
Cell lysate
PMA
BAPTA-AM
- + +
- - +
RalA-GTP
RalB-GTP

Figure 22

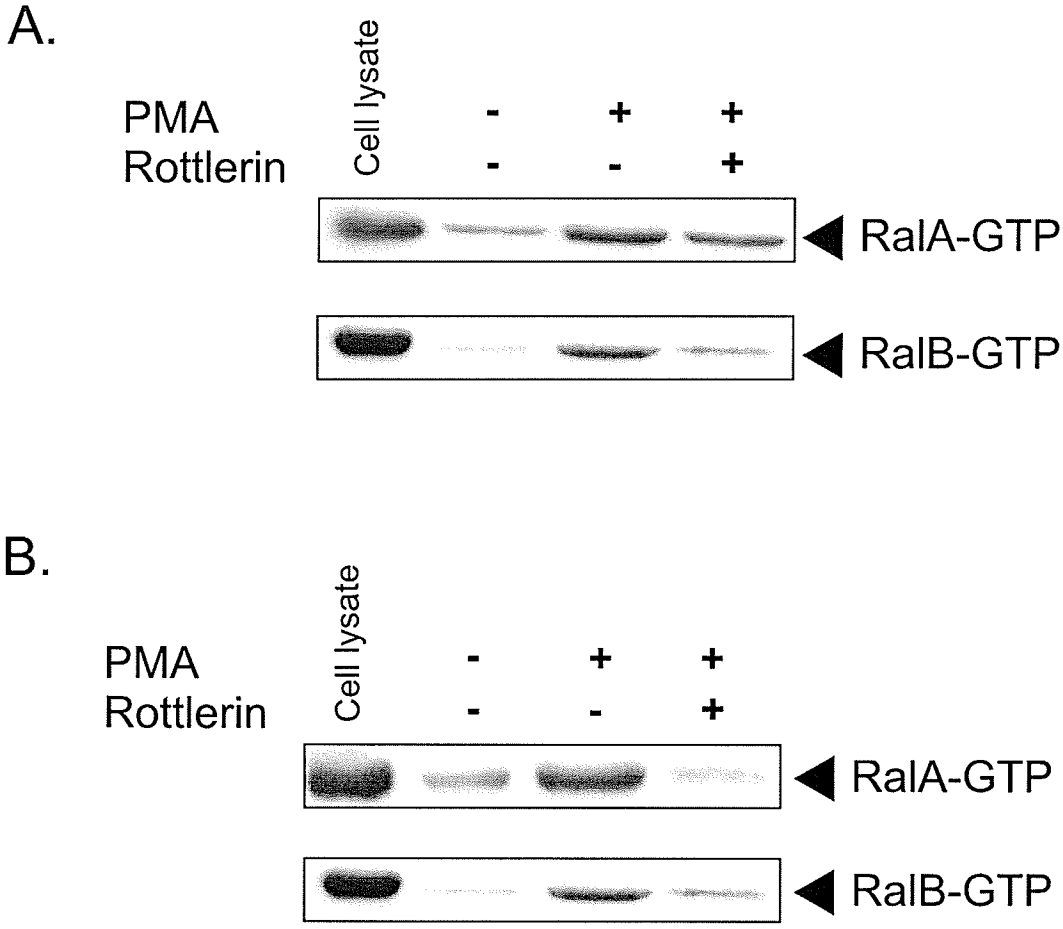

A.
Cell lysate
PMA
Rottlerin
-
+
+
-
-
+
RalA-GTP
RalB-GTP
B.
Cell lysate
PMA
Rottlerin
-
+
+
-
-
+
RalA-GTP
RalB-GTP

Figure 23

Fig. 23. PMA-induced activation of Ral occurs, in part, via PKCδ. Quiescent, subconfluent A7r5 (A) and HeLa (B) cells were treated with 10 μM Rottlerin prior to a 5 minute treatment with 1 μM PMA, and active Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA (A and B, upper panels) or –RalB (A and B, lower panels) antibodies. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown). The experiments were repeated at least three times and gave identical results.

activates RalA and RalB in a Ca^{2+}/CaM-dependent manner, and that the δ isoform of PKC is largely involved.

5.2.6 Ral Mutants are Abundantly Expressed in Transfected A7r5 and HeLa Cells

In each transfection experiment to follow in which A7r5 and HeLa cells were transfected with pRK-5[RalA(V23)], pRK-5[RalB(V23)], pRK-5[RalB(N28)], pRK-5, pSSRαII($p130^{Cas}$) or pFLAG-CMV2[RalGPS1B], the protein expression of each transfected cDNA was verified. Western blotting with appropriate antibody demonstrated that the amount of total RalA, RalB, $p130^{Cas}$ and FLAG-tagged RalGPS1B in transfected A7r5 (examples in Fig. 24) and HeLa (examples in Fig. 25) cells, including the $p130^{Cas}$ and RalGPS1B doubly-transfected HeLa cells, was substantially greater than that in control and pRK-5-transfected cells. Results also show that transfected DNA was equally expressed as protein in A7r5 and HeLa cells.

5.2.7 Ras-dependent Ral-GEFs are Required for Basal and EGF-induced Activation of RalA in A7r5 but not HeLa Cells

Ral can be activated by Ras-dependent and Ras-independent pathways. Ras-independent pathways include Ca^{2+}/CaM-dependent and Ras-independent RalGEF (Rebhun *et al.*, 2000; Gotoh *et al.*, 2000) pathways. Therefore, to examine the role of Ras-dependent and Ras-independent RalGEFs in EGF-induced activation of RalA, A7r5 and HeLa cells were transfected with dominant-negative RalB(N28) or empty pRK-5 plasmid, treated 24 hours later with EGF, and activated Ral isolated by GST-RRBD pull-down assay. RalB(N28) mops up Ras-dependent RalGEFs making them unavailable to

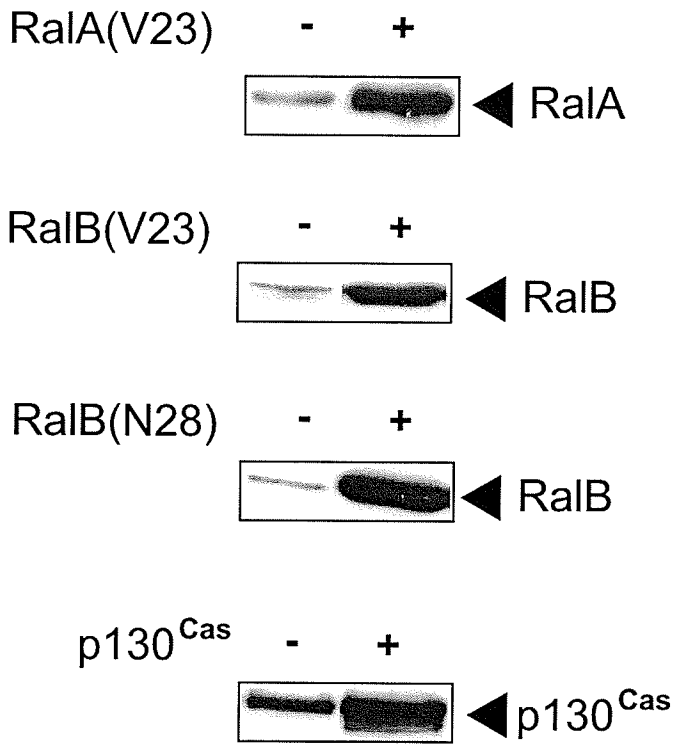


Figure 24

Fig. 24. Ral mutants and CAS are abundantly expressed in transfected A7r5 cells. Transfected and untransfected control A7r5 cells were lysed in Laemmli sample buffer 18 hours after transfection with pRK-5[RalA(V23)], pRK-5[RalB(V23)], pRK-5[RalB(N28)], pRK-5, or pSSRαII($p130^{Cas}$), briefly sonicated, separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA , -RalB, or - $p130^{Cas}$ antibodies to detect total RalA, RalB and $p130^{Cas}$ protein expression. Transfection was verified for each transfection experiment performed.

Fig. 25. Ral mutants, $p130^{Cas}$ and RalGPS1B are abundantly expressed in transfected HeLa cells. Transfected and untransfected control HeLa cells were lysed in Laemmli sample buffer 18 hours after transfection with pRK-5[RalA(V23)], pRK-5[RalB(V23)], pRK-5[RalB(N28)], pRK-5, pSSRαII($p130^{Cas}$) or pFLAG-CMV2[RalGPS1B], briefly sonicated, separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA , -RalB, - $p130^{Cas}$ or -FLAG antibodies to detect total RalA, RalB, $p130^{Cas}$ and RalGPS1B protein expression. Transfection was verified for each transfection experiment performed.

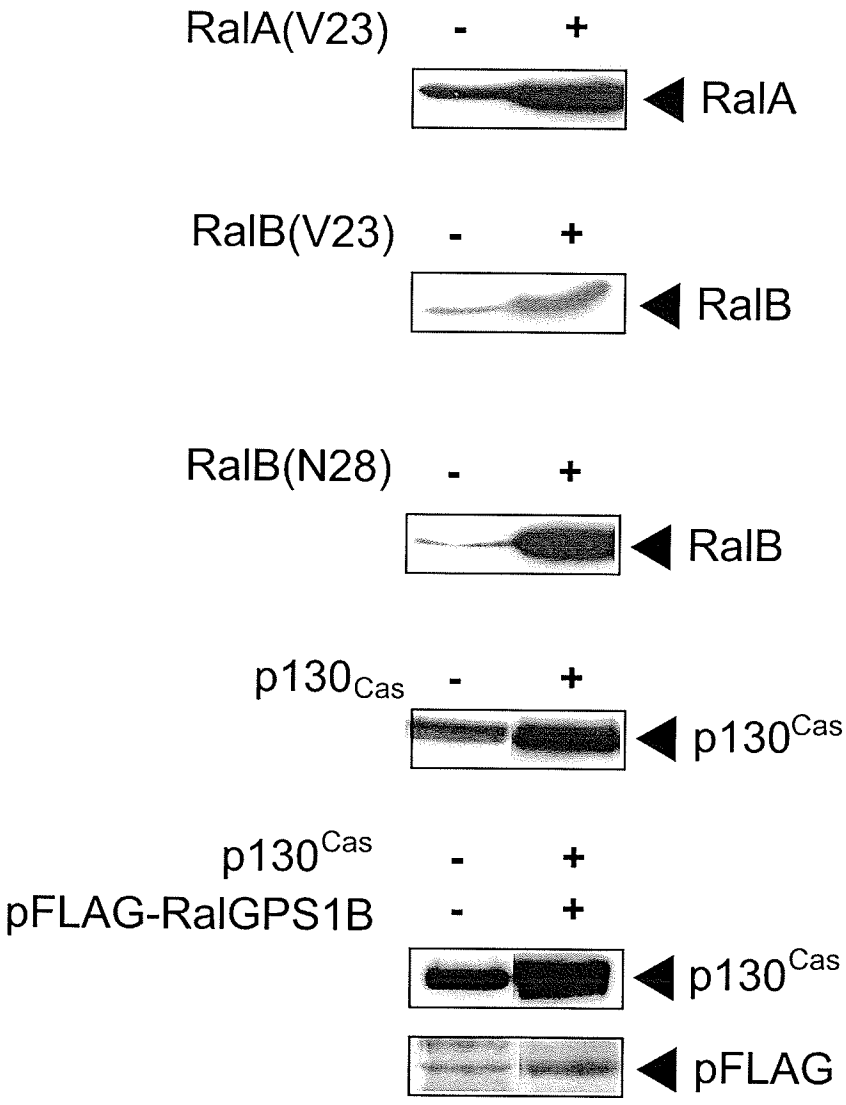

RalA(V23) - +
RalA
RalB(V23) - +
RalB
RalB(N28) - +
RalB
p130Cas - +
p130Cas
p130Cas - +
pFLAG-RalGPS1B - +
p130Cas
pFLAG

Figure 25

activate Ral. Transfection of A7r5 cells with RalB(N28) (Fig. 26A, upper panel) resulted in a decrease in both basal and EGF-induced RalA activation, the latter to below basal levels. By contrast in HeLa cells, basal activation was increased by, and EGF-induced RalA activation was determined by quantification to be unaffected by dominant-negative Ral (Fig. 26A, lower panel). The inhibition of EGF-induced Ral activation in RalB-(N28)-transfected A7r5 cells (Fig. 26B, upper panel; 54% +/- 3% SD inhibition) was enhanced about 22% by the addition of W7 (Fig. 26B, upper panel; 76% +/- 3% SD inhibition). However, quantification determined that both RalB(N28) and W7 had no effect on the EGF-induced activation of RalA in HeLa cells (Fig. 26B, lower panel). These results suggest that the Ras/RalGEF pathway and CaM are required for the EGF-induced activation of RalA in A7r5 but not HeLa cells.

5.2.8 The Ras-independent Ral-GEF, BCAR3/AND-34, is Required for the EGF-induced Activation of RalA in HeLa but not A7r5 Cells, and BCAR1/p130^{Cas} is Stimulatory to Ral Activation in MCF7 cells

Because Ral can be activated via RalGEFs independently of Ras (Rebhun *et al.*, 2000; Gotoh *et al.*, 2000), we wished to determine if the Ras-independent RalGEF, BCAR3/AND-34 is required for EGF-induced Ral activation. The binding of p130^{Cas} to the GEF domain of AND-34 inhibits the ability of AND-34 to promote Ral-GTP levels in cells (Gotoh *et al.*, 2000). Therefore, we transfected A7r5, MCF7 and HeLa cells with p130^{Cas} and monitored Ral activation in control and transfected cells treated with EGF using GST-RRBD pull-down assays (Fig. 27). In A7r5 cells, quantification showed that p130^{Cas} transfection had no effect on EGF-induced RalA (Fig. 27A, upper panel) or RalB

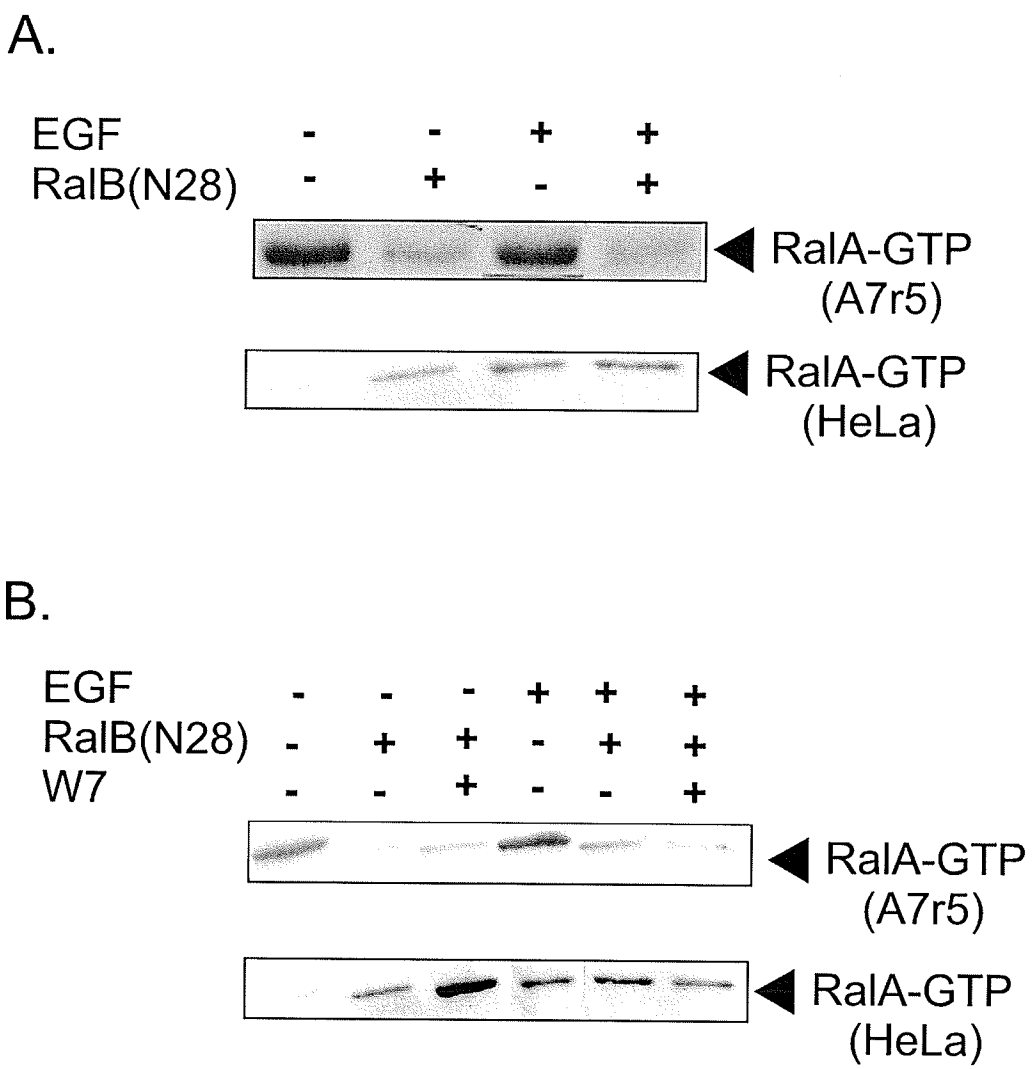

A.
EGF - - + +
RalB(N28) - + - +
RalA-GTP (A7r5)
RalA-GTP (HeLa)
B.
EGF - - - + + +
RalB(N28) - + + - + +
W7 - - + - - +
RalA-GTP (A7r5)
RalA-GTP (HeLa)

Figure 26

Fig. 26. Ras-dependent Ral-GEFs are required for the EGF-induced activation of RalA in A7r5 but not HeLa cells. Quiescent, subconfluent A7r5 and HeLa cells (A, B) were transfected with RalB(N28) 24 hours prior to a 15 minute treatment with 10 ng/ml recombinant EGF. Some cells (B, upper and lower panels) were treated with 50 μM W7 30 minutes before the EGF treatment. As described in Methods, activated Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA antibody. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown. To verify successful transfection, separate aliquots were probed with ant-RalB antibody to measure total RalB expression (results not shown). The experiments were repeated at least three times and gave identical results.

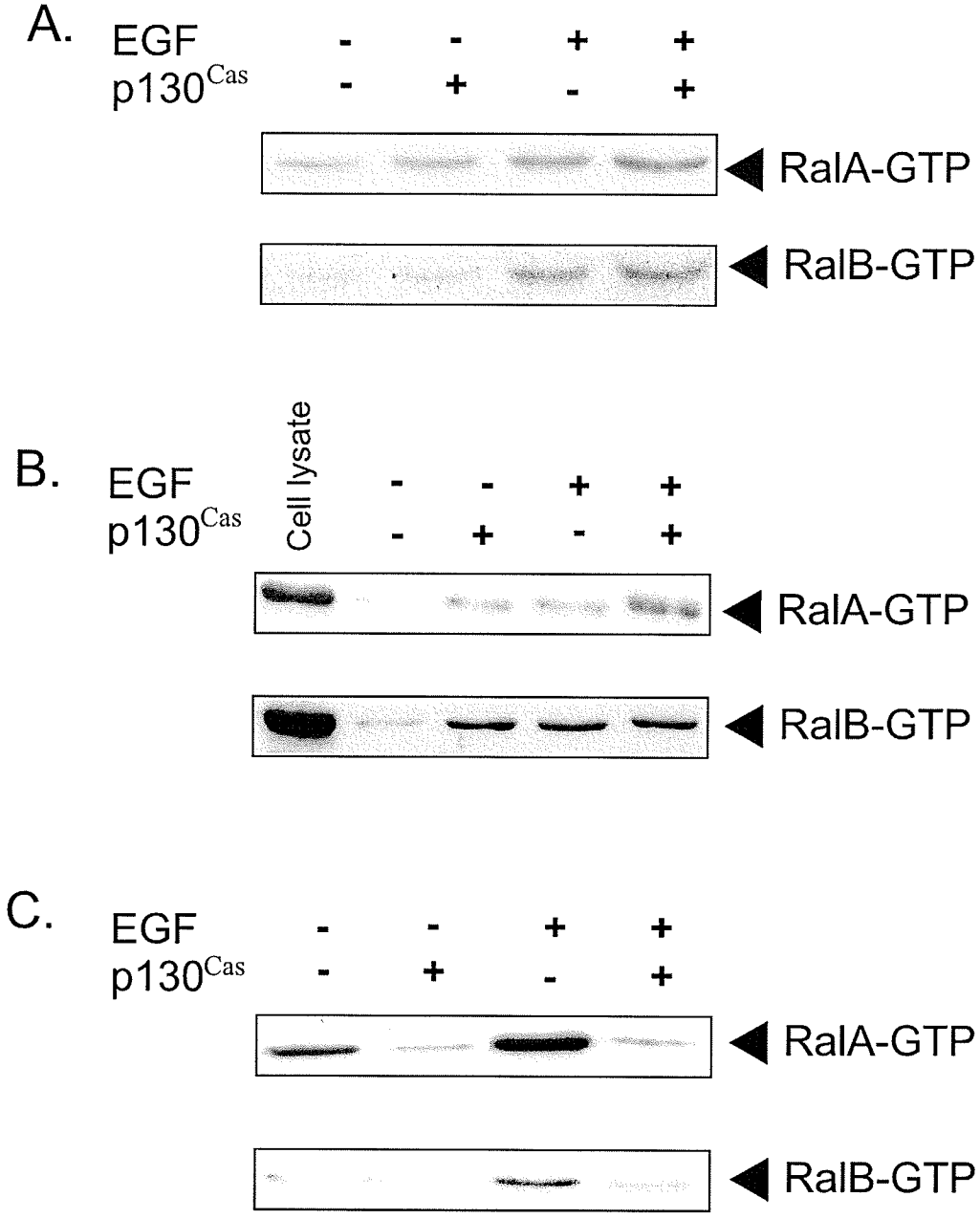

A.
EGF
p130Cas
- - + +
- + - +
RalA-GTP
RalB-GTP
B.
Cell lysate
EGF
p130Cas
- - + +
- + - +
RalA-GTP
RalB-GTP
C.
EGF
p130Cas
- - + +
- + - +
RalA-GTP
RalB-GTP

Figure 27

Fig. 27. The Ras-independent Ral-GEF, BCAR3/AND-34, is required for the EGF-induced activation of RalA in HeLa but not A7r5 cells, and BCAR1/p130^{Cas} is stimulatory to Ral activation in MCF7 cells. Quiescent, subconfluent A7r5 (A), MCF7 (B) and HeLa (C) cells were transfected with p130^{Cas} 24 hours prior to a 15 minute treatment with 10 ng/ml recombinant EGF. As described in Methods, activated Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA or -RalB antibodies. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown). To verify successful transfection, separate aliquots were also probed with anti- p130^{Cas} antibody (results not shown). The experiments were repeated at least three times and gave identical results.

(Fig. 27A, lower panel) activation. In MCF7 cells, $p130^{Cas}$ transfection alone increased RalA (Fig. 27B, upper panel) and RalB (Fig. 27B, lower panel) activation above basal levels. There was an increase in EGF-induced RalA activation in $p130^{Cas}$ transfectants (Fig. 27B, upper panel; 30% +/- 11% SD), but no increase in EGF-induced RalB activation. By contrast, in HeLa cells $p130^{Cas}$ markedly inhibited EGF-induced RalA (Fig. 27C, upper panel; 89% +/- 2.5% SD) and RalB (Fig. 27C, lower panel; 93% +/- 1% SD) activation. To determine whether $p130^{Cas}$ can also inhibit RalGPS, HeLa cells were co-transfected with $p130^{Cas}$ and FLAG-tagged RalGPS1B 24 hours before EGF treatment and pull-down of activated Ral by GST-RRBD. $p130^{Cas}$ did not inhibit but appeared to enhance EGF/RalGPS1B-mediated RalA activation (Fig. 28A). We had previously determined that $p130^{Cas}$ transfection does not affect total Ral protein expression (Fig. 28B). There was no difference in Ral activation in Hela cells stimulated with EGF and in cells transfected with RalGPS1B and stimulated with EGF (Fig. 28A). This suggests that RalGPS1B must not operate in HeLa cell RalA activation. Our results point to the novel finding that in HeLa cells, EGF activates Ral primarily via the Ras-independent RalGEF, BCAR3, while in A7r5 cells EGF activates Ral primarily via the Ras-dependent RalGEFs. Results also demonstrate that the actions of $p130^{Cas}$ depend on cell type and cell environment. In contrast to our findings in HeLa cells, $p130^{Cas}$ is stimulatory to basal and EGF-induced Ral activation in MCF7 cells (compare Fig. 27B and 28A) and to RalA activation in HeLa cells over-expressing RalGPS1B and treated with EGF.

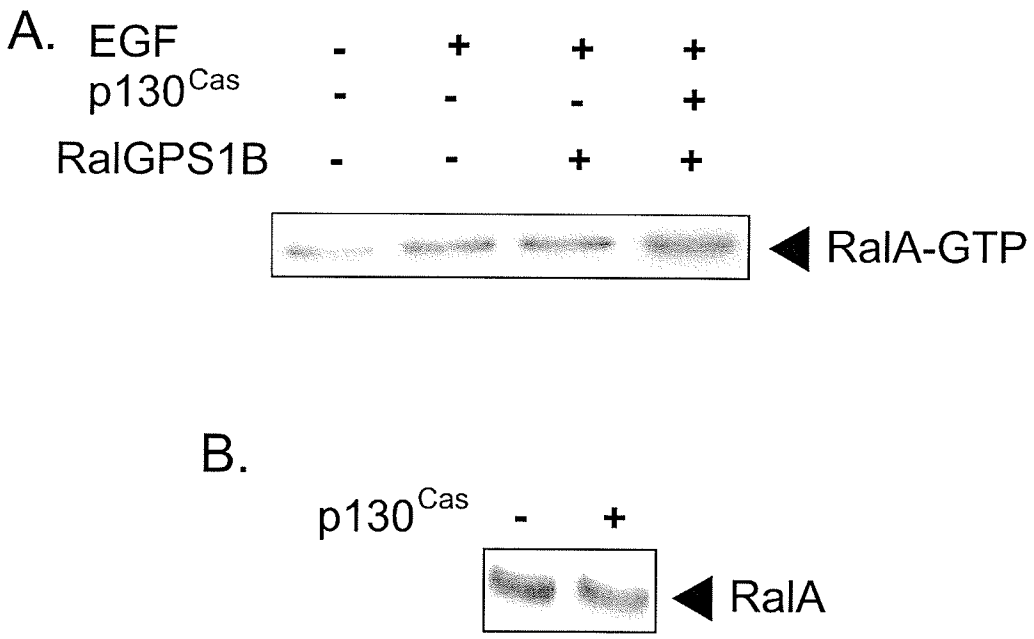


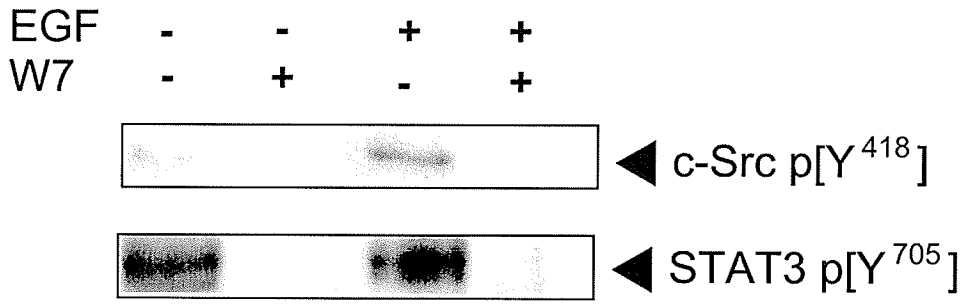
Figure 28

Fig. 28. BCAR1/p130^{Cas} stimulates EGF-induced Ral activation in HeLa cells over-expressing RalGPS1B. HeLa cells (A) were co-transfected with RalGPS1B and p130^{Cas} 24 hours prior to a 15 minute treatment with 10 ng/ml recombinant EGF. As described in Methods, activated Ral was pulled down from cell lysates with GST-RRBD coupled to GSH-agarose beads, proteins boiled off beads, separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-RalA antibody. Some p130^{Cas}-transfected HeLa cells (B) were also probed with anti-RalA to detect total RalA. To ensure equal protein loading, aliquots were taken from cell lysates before beads were added and probed with anti-β-actin (results not shown). To verify successful transfection, separate aliquots were also probed with anti- p130^{Cas} and –FLAG antibodies (results not shown). The experiments were repeated at least three times and gave identical results.

5.2.9 EGF-induced Activation of c-Src and STAT3 Requires CaM but not Ca^{2+} in A7r5 and HeLa Cells

Because c-Src and STAT3 are activated by EGF through Ral (Goi *et al.*, 2000), it was next examined whether the EGF-induced activation of c-Src and STAT3 also requires Ca^{2+} and CaM. Quiescent HeLa and A7r5 cells were stimulated with EGF in the presence or absence of W7, BAPTA-AM or U73122, and cell lysates immunoblotted with anti-c-Src p[Y^{418}] or -STAT3 p[Y^{705}] polyclonal antibodies. Monoclonal anti-nonphosphorylated c-Src and STAT3 antibodies were used as controls. In quiescent A7r5 (Fig. 29A) and HeLa (Fig. 29B) cells, W7 eliminated both basal and EGF-induced c-Src (Fig. 29A and B, upper panels) and STAT3 (Fig. 29A and B,lower panels) activation, whereas BAPTA-AM had no effect in A7r5 (Fig. 30A) and HeLa (Fig. 30B) cells on EGF-induced c-Src (Fig. 30A and B, upper panels) or STAT3 (Fig. 30A and B, lower panels) activation. U73122 was also shown to inhibit EGF-induced c-Src (Fig. 30A, upper panel) and STAT3 (Fig. 30A, lower panel) activation in A7r5 cells. These results indicate that in A7r5 and HeLa cells, basal and EGF-induced c-Src and STAT3 activation is CaM-dependent but Ca^{2+}-independent. EGF-induced c-Src and STAT3 activation is PLC-dependent in A7r5 cells, potentially through the generation of DAG.

A.



B.

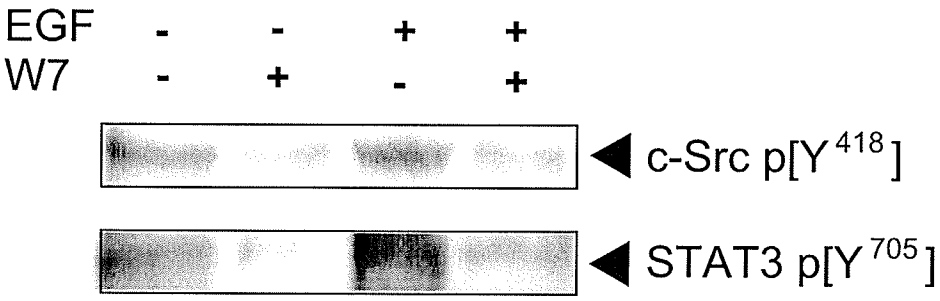


Figure 29

Fig. 29. EGF-induced activation of c-Src and STAT3 depends on CaM in A7r5 and HeLa cells. Quiescent, subconfluent A7r5 (A) and HeLa (B) cells were treated for 30 minutes with 50 μM W7 prior to a 15 min treatment with 10 ng/ml recombinant EGF. As described in Methods, cells were lysed in RIPA buffer, proteins separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-c-Src p[Y^{418}] (A and B, upper panels) or –STAT3 p[Y^{705}] (A and B, lower panels) polyclonal antibodies. To ensure equal protein loading, separate aliquots were taken from cell lysates and probed with anti-β-actin (results not shown). The experiments were repeated at least three times and gave identical results.

Fig. 30. EGF-induced activation of c-Src and STAT3 depends on PLC in A7r5 cells, but does not require Ca^{2+} in A7r5 and HeLa cells. Quiescent, subconfluent A7r5 (A) and HeLa (B) cells were treated for 30 minutes with 50 μM W7 or BAPTA-AM prior to a 15 minute treatment with 10 ng/ml recombinant EGF. Some A7r5 cells (A) were treated for 5 minutes with 10 μM U73122 prior to EGF treatment. As described in Methods, cells were lysed in RIPA buffer, proteins separated by 13% SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-c-Src p[Y418] (A and B, upper panels) or –STAT3 p[Y705] (A and B, lower panels) polyclonal antibodies. To ensure equal protein loading, separate aliquots were taken from cell lysates and probed with anti-β-actin (results not shown). The experiments were repeated at least three times and gave identical results.

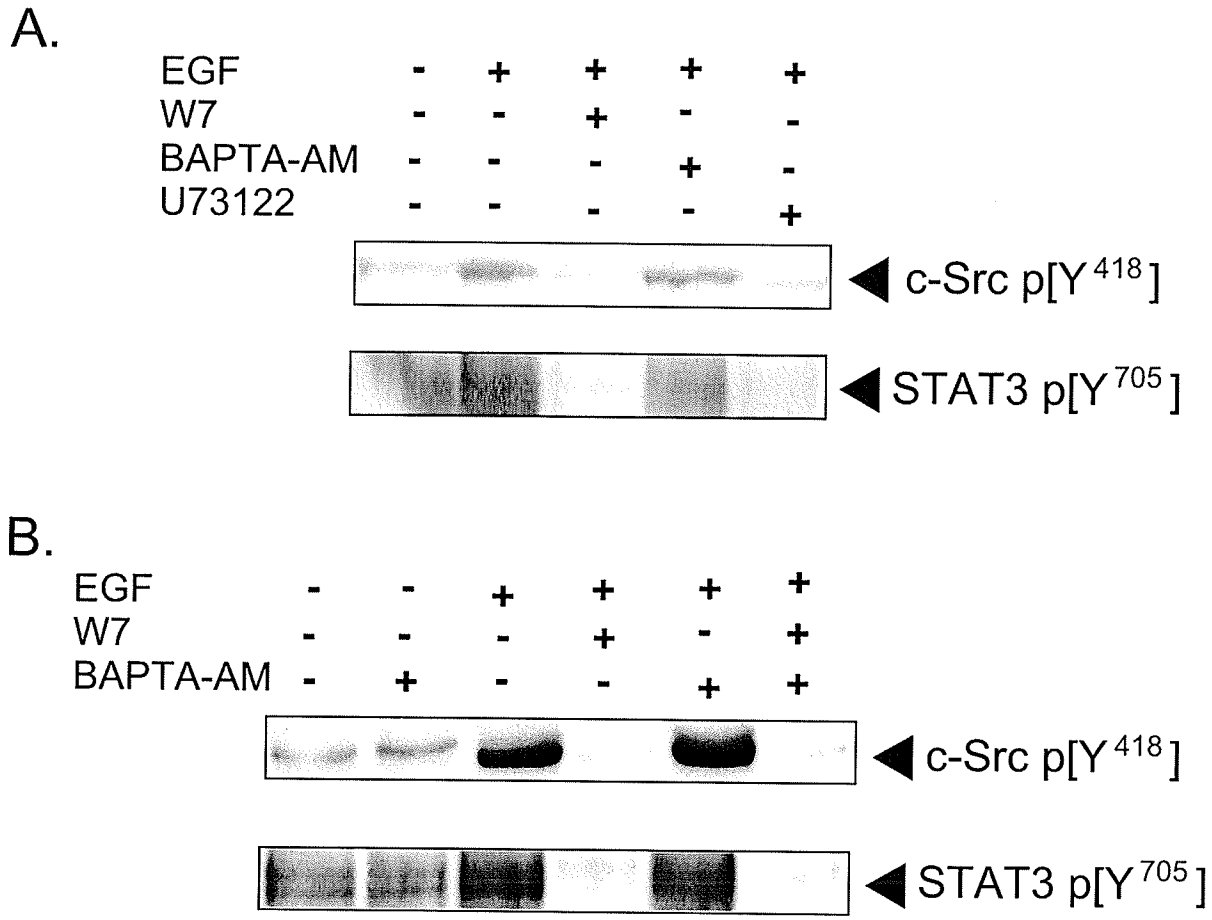

A.
EGF - + + + +
W7 - - + - -
BAPTA-AM - - - + -
U73122 - - - - +
c-Src p[Y 418]
STAT3 p[Y 705]
B.
EGF - - + + + +
W7 - - - + - +
BAPTA-AM - + - - + +
c-Src p[Y 418]
STAT3 p[Y 705]

Figure 30

5.3 Ral Interacts Directly With the β1 Subunit of Na^+/K^+-ATPase, and is Involved in the Function of Na^+/K^+-ATPase

5.3.1 β1 subunit of Na^+/K^+-ATPase Interacts with RalA and RalB *in vivo*

Similar to cardiac glycosides, both Ral and RalGDS (when over-expressed) upregulate proto-oncogenes and cardiac hypertrophic genes (Kawai *et al*, 2002). Therefore, we suspect Ral is involved in the ouabain-stimulated signal transduction pathways of Na^+/K^+-ATPase. To study this, and to determine additional functions for Ral-GTPases, a human testis cDNA expression library was screened by a yeast two-hybrid assay for novel proteins interacting directly with RalB. Positive blue colonies are caused by the protein-protein-induced coupling of the BD and AD of the GAL4 transcription factor, which results in activation of the yeast MEL1 gene, stimulation of α-galactosidase synthesis and digestion of X-α-Gal. Blue colonies produced in the presence of X-α-Gal resulted when Y187 cells transfected with pACT2[testis library cDNAs] were mated with AH109 cells transfected with pGBKT7[RalB]. One of several positive clones isolated was found to be highly identical to the β1 subunit of Na^+/K^+-ATPase. This cDNA was shown on sequencing to be missing the first 350 5' base pairs, comprising the cytoplasmic and transmembrane domains. To determine if RalB interacts directly with full-length β1 in a eukaryotic system, Y2H assays testing interaction between recombinant BD-RalA and BD-RalB fusion proteins with recombinant AD-full length β1 (Fig. 31A) showed both Rals interacted directly with β1 *in vivo* in this system. The reaction was specific because the AD[β1] (Fig. 31A) and BD[Ral] (Fig. 11, and Clough *et al.*, 2002) constructs do not autonomously turn yeast colonies blue. We have previously shown above, by the use of positive and negative controls, that in our hands

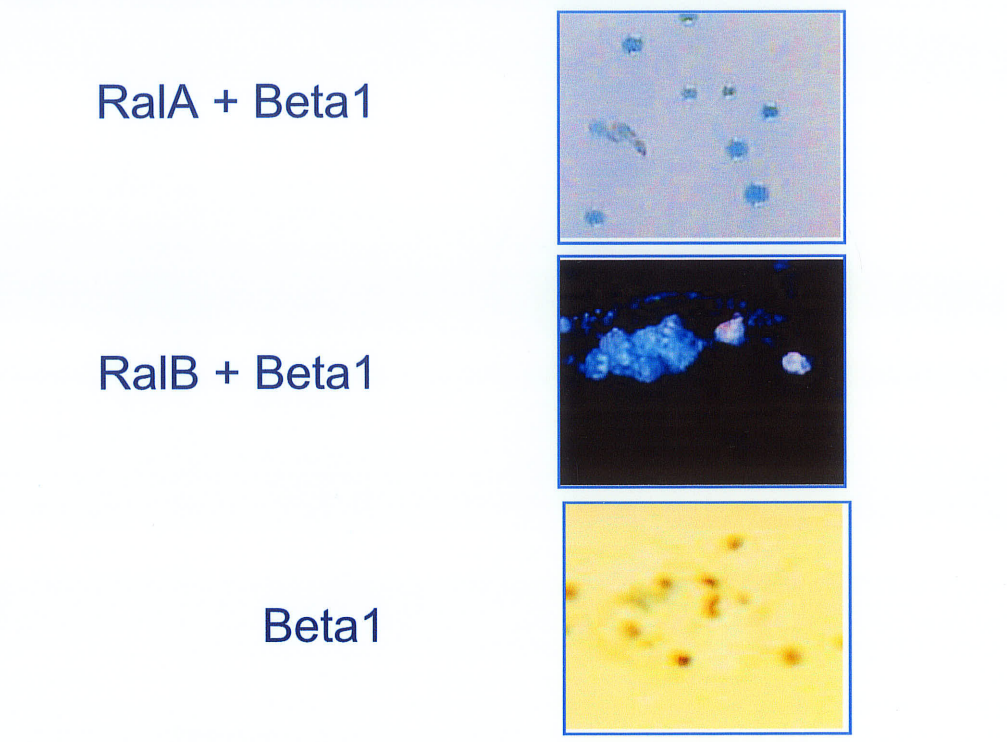

A.
RalA + Beta1
RalB + Beta1
Beta1
B.
RalA
RalB
Lysate
BD.cMyc.Ral
C.
AH109 + Beta1
Y187 + Beta1
AH109
Y187
AD.HA.Beta1

Figure 31

Fig. 31. β1 subunit of Na^+/K^+-ATPase interacts with RalA and RalB in vivo. (A) RalA and RalB cDNAs were subcloned into GAL4 BD expression plasmid pGBKT7, and β1 cDNA was subcloned into GAL4 AD expression plasmid pGADT7. To identify specific protein-protein interactions, yeast two hybrid assays were performed as described in Methods. Colonies were monitored for blue coloration caused by the direct interaction between the GAL4 BD and AD fusion proteins. Interactions tested were pGBKT7[RalA] and pGADT7[β1] (RalA + β1), and pGBKT7[RalB] and pGADT7[β1] (RalB + β1). pGADT7[β1] (β1) was also tested for autonomous activation of the MEL1 gene. In the positive cases, the positive phenotype persisted upon restreaking at least 3 times onto high stringency drop-out medium. (B, C) Transformed yeast cells express BD[Ral] and AD[β1] fusion proteins. AH109 yeast cells were transformed with pGBKT7[RalA] or pGBKT7[RalB] (B), or with pGADT7[β1] (C), and Y187 yeast cells were transformed with pGADT7[β1] (C). Proteins were extracted from transformed and untransformed control yeast as described in Methods, separated by 13% SDS-PAGE, transferred to PVDF membranes and probed with anti-RalA, -RalB or -β1 antibodies.

this system is specific (Fig. 11, and Clough *et al.*, 2002). We also verified by western blotting that BD[Ral] (Fig. 31B) and AD[β1] (Fig. 31C) fusion proteins were expressed in transformed AH109 and Y187 yeast strains.

5.3 2 Na^+/K^+-ATPase is Present in Both Cytosol and Membranous Fractions of Human Platelets

To determine the distribution of Na^+/K^+-ATPase in human platelets, western blotting using anti-β1 Na^+/K^+-ATPase monoclonal antibody was performed on cytosol and membranous fractions of platelets, as well as total platelet extracts. Both cytosolic and particulate fractions of platelets (Fig. 32. upper panel) were shown to contain β1. The total rat kidney lysate (Fig. 32, lower panel) was used as a positive control. That β1, a membrane protein, was detected in cytosol, suggests that this fraction may have been contaminated with membranes, or that β1 may also occur as a soluble protein.

5.3.3 Verification that GST-β1 Fusion Proteins are Expressed in *E. coli*

Before proceeding with *in* vitro binding experiments using GST-β1 fusion proteins, it was verified by Coomassie staining and western blotting (Fig. 33) that competent bacterial BL21-CodonPlus RIL cells transformed with pGEX4T2[β1] could express GST-β1 fusion protein. Therefore, as described in Methods, bacterial transformants were stimulated with IPTG, cells lysed, proteins separated by 13% SDS-PAGE and the gel stained with Coomassie blue. The proteins were transferred to PVDF membrane and blotted with anti-β1 antibody. When expressed in DH5α (results not shown) or AD202 cells, GST-β1 consistently showed two bands approximating the mass

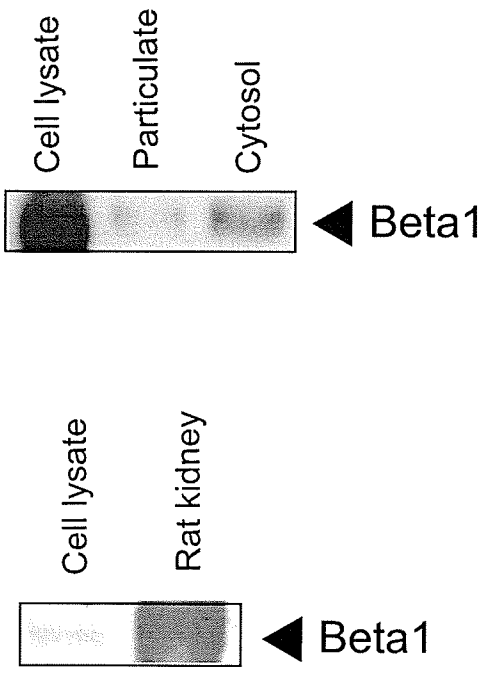

Cell lysate
Particulate
Cytosol
Beta1
Cell lysate
Rat kidney
Beta1

Figure 32

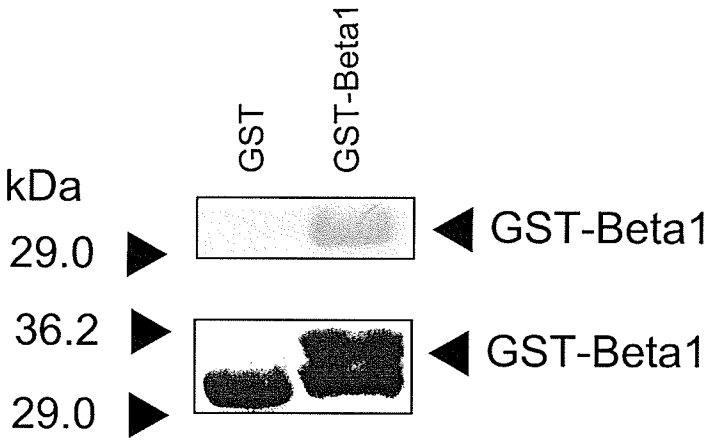
Fig. 32. Na^+/K^+-ATPase is present in both cytosol and membranous fractions of human platelets. Outdated human platelets (upper and lower panels) were prepared and fractionated into cytosolic and membranous fractions, as described in Methods. Total rat kidney lysate (lower panel) was used as a positive control. Samples were solubilized in Laemmli's buffer, and proteins separated by 13% SDS-PAGE, transferred to PVDF membranes and probed with anti-β1 Na^+/K^+-ATPase monoclonal antibody.

of GST and a slightly heavier protein on western blotting (Fig. 33A, upper panel) and Coomassie blue staining (Fig. 33A, lower panel). AD202 cells are a protease-deficient strain of *E. coli*. Therefore, the fragmented proteins were suspected of being caused by codon bias, resulting in a very short truncated β1 product. Therefore, BL21-CodonPlus RIL cells, an *E. coli* strain that overcomes codon bias, were transformed with pGEX4T2[β1]. Both western blotting (Fig. 33B, upper panel) and Coomassie staining (Fig. 33B, lower panel) showed an identically sized protein of mass equivalent to full length GST-β1 fusion protein (55-60 kDa) was expressed. Staining, but not western analysis (results not shown), also showed the 30-35 kDa sized bands. This was proof that the smaller bands represented GST and GST-truncated β1, in which none or only a small part of β1 was being synthesized, a region N-terminal to the epitope recognized by the β1 antibody.

5.3.4 Endogenous β1 (or endogenous RalB) in Human Platelets Interacts with GST-RalA and –RalB, plus Sepharose-RalA and-RalB (or GST-β1) *In Vitro*

To determine if endogenous human platelet β1 subunit of Na^+/K^+-ATPase interacts with Ral *in vitro*, proteins pulled down from total platelet lysates by GSH-agarose-coupled GST-RalA and GST-RalB fusion proteins, or GST as negative control, were probed with anti-β1 Na^+/K^+-ATPase monoclonal antibody (Fig. 34A). Results show that both GST-RalA and –RalB, but not GST, interact with endogenous β1 in human platelets. In the reverse reaction, to determine if endogenous RalB from human platelets interacts with β1 *in vitro*, proteins pulled down from total platelet lysates by GSH-agarose-coupled GST-β1 or GST were probed with anti-RalB polyclonal antibody (Fig.

A.


GST
GST-Beta1
kDa
29.0
GST-Beta1
36.2
29.0
GST-Beta1

B.

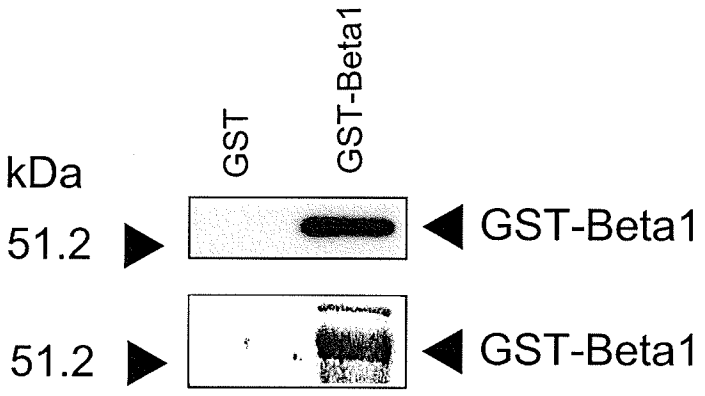

GST
GST-Beta1
kDa
51.2
GST-Beta1
51.2
GST-Beta1

Figure 33

Fig. 33. GST-β1 fusion proteins are expressed in *E. coli* strain BL21-CodonPlus RIL. AD202 (A) and BL21-Codon Plus RIL (B) *E. coli* strains were transformed with pGEX4T2[β1] or pGEX-4T constructs. GST-*β*1 or GST proteins were stimulated to be expressed by IPTG as described in Methods, separated by 13% SDS-PAGE, stained with Coomassie blue (A, B, lower panels), transferred to PVDF membrane and blotted with anti-β1 antibody (A, B, upper panels).

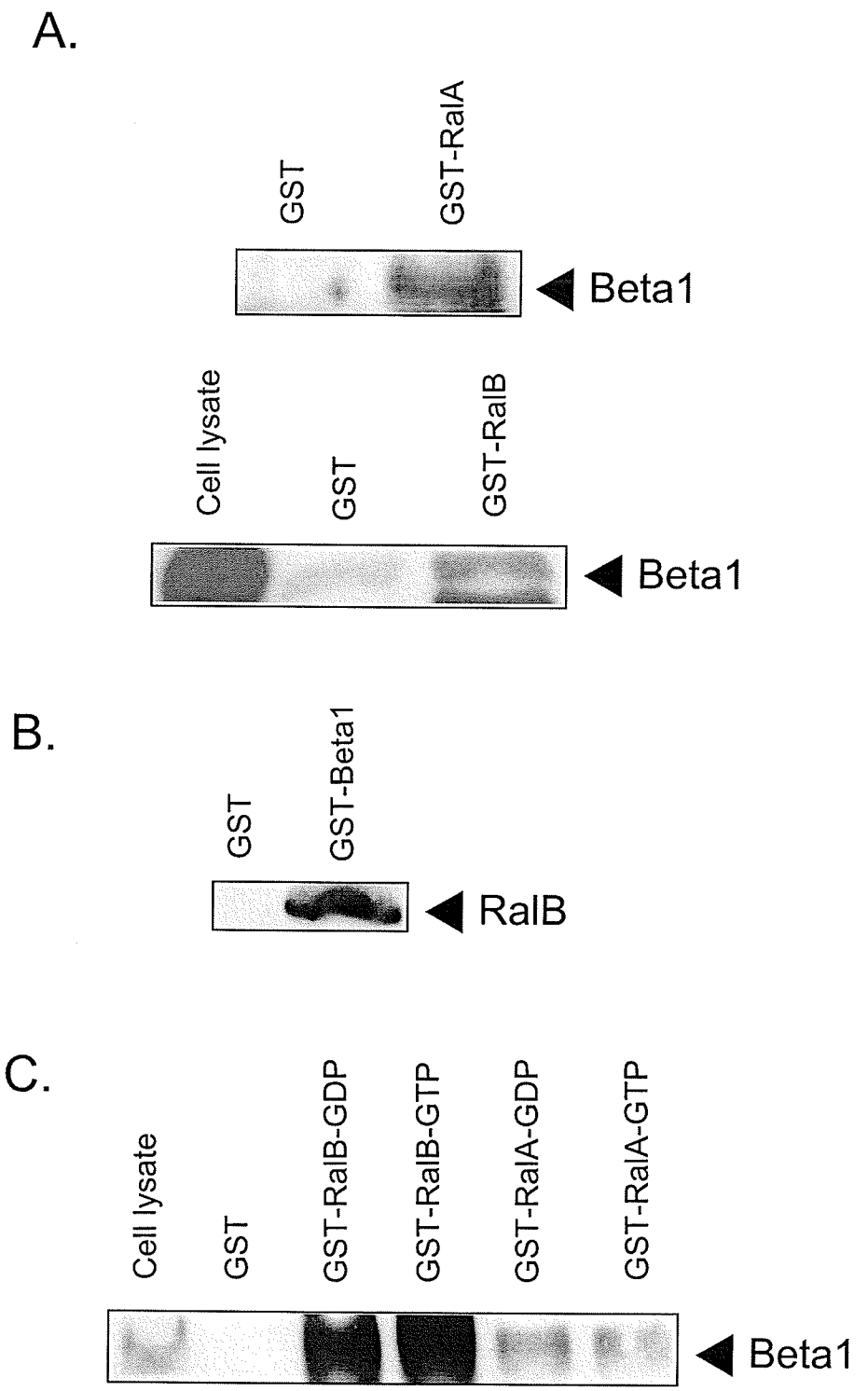

A.
GST
GST-RalA
Beta1
Cell lysate
GST
GST-RalB
Beta1
B.
GST
GST-Beta1
RalB
C.
Cell lysate
GST
GST-RalB-GDP
GST-RalB-GTP
GST-RalA-GDP
GST-RalA-GTP
Beta1

Figure 34

Fig. 34. Endogenous β1 subunit of Na^+/K^+ATPase (or endogenous RalB) in human platelets interacts with GST-Ral (or GST- β1) *in vitro*, independently of Ral nucleotide status. (A) Endogenous β1 subunit of Na^+/K^+ATPase in human platelets interacts with GST-Ral *in vitro*. Outdated human platelets were prepared as described in Methods, lysed in Ral buffer and incubated with 50 μl GSH-agarose beads coupled to GST-RalA or –RalB, or GST as negative control. Bound proteins were boiled off the beads, solubilized in Laemmli buffer, separated by 13% SDS-PAGE, transferred to PVDF membranes and probed with anti-β1 Na^+/K^+-ATPase monoclonal antibody. (B) Endogenous RalB in human platelets interacts with GST-β1 subunit of Na^+/K^+ATPase *in vitro*. In an identical experiment, outdated human platelet lysates were incubated with 50 μl GSH-agarose-coupled GST-β1 or GST, and bound proteins separated and probed with anti-RalB polyclonal antibody. (C) The Ral/β1 interaction occurs independently of Ral nucleotide status. In an identical experiment, outdated human platelet lysates were incubated with 50 μl GSH-agarose-coupled GST-RalAGTP-γS, -RalAGDP-βS, -RalBGTP-γS or –RalBGDP-βS, or GST, and bound proteins separated and probed with anti-β1 monoclonal antibody. All experiments were repeated at least three times and gave identical results.

34B). Results show that GST-β1, but not GST, bound to endogenous RalB. To determine if this interaction depends on nucleotide status, proteins pulled down from total platelet lysates by GSH-agarose-coupled GST-RalA/GTP-γS, GST-RalA/GDP-βS, GST-RalB/GTP-γS, and GST-RalB/GDP-βS, or GST as negative control, were probed with anti-β1 Na^+/K^+-ATPase monoclonal antibody (Fig. 34C). Results demonstrate that both GTP- and GDP-bound GST-Ral proteins, but not GST, interacted with β1. In identical experimental protocols, Sepharose-CNBr-coupled RalA and RalB, but not Sepharose control beads, (Fig. 35A), pulled down endogenous β1 in human platelets. Results therefore show that both active and inactive Ral bind β1 *in vitro* in human platelets.

5.3.5 Sepharose-CNBr-Coupled RalA and RalB (or β1) Interact *In Vitro* with Endogenous β1 (or RalA and RalB) in Canine Sarcolemmal Membranes

To demonstrate RalA and RalB interact *in vitro* with endogenous β1 in other tissues, a Sepharose-fusion protein pull-down experiment was performed using enriched CSMs (Fig. 35). Sepharose-CNBr-coupled RalA and RalB, but not Sepharose control beads, interacted with endogenous β1 in CSMs (Fig. 35B). In the reverse reaction, endogenous RalA (Fig. 35C, upper panel) and RalB (Fig. 35C, lower panel) were shown to interact with Sepharose-CNBr-coupled β1. These results further confirm that Ral and β1 interact *in vitro*, and in more than one species and tissue type.

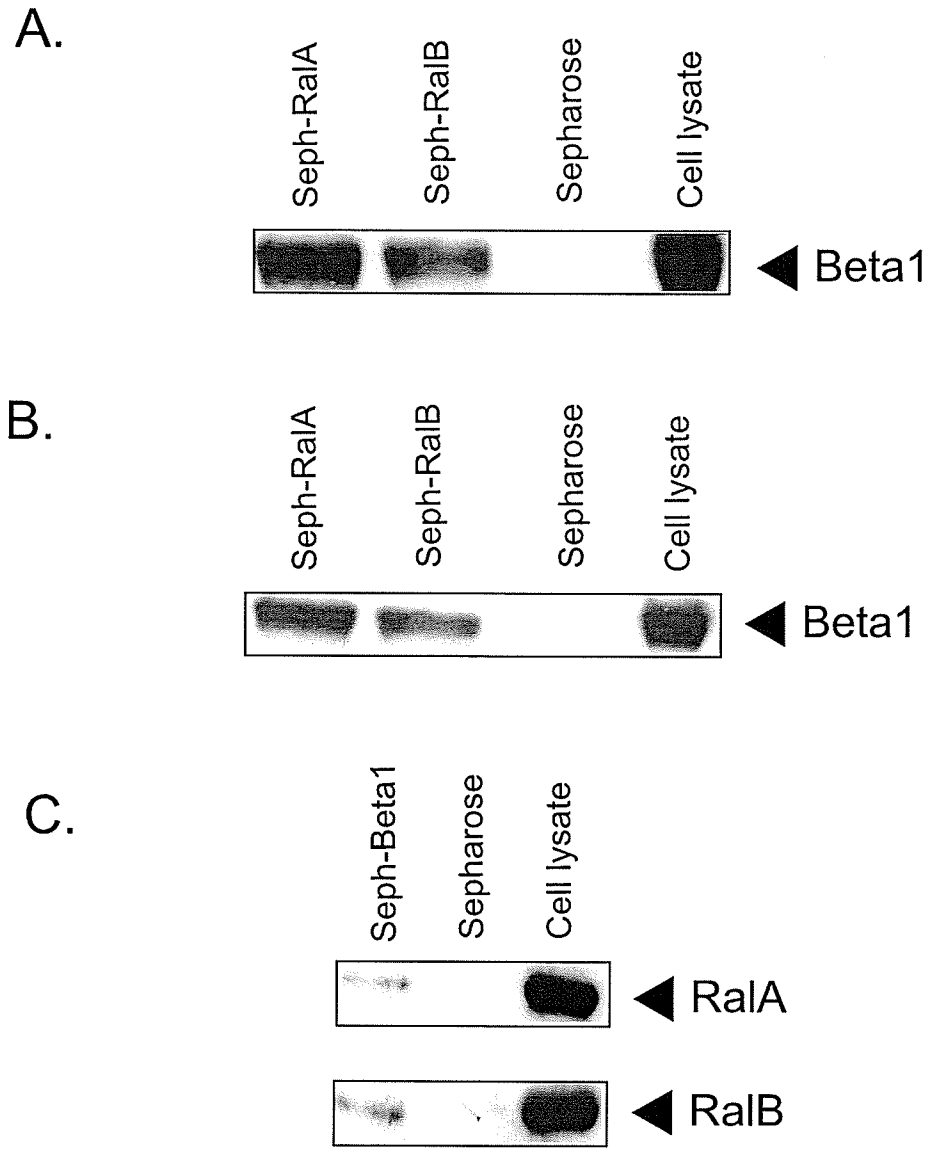

A.
Seph-RalA
Seph-RalB
Sepharose
Cell lysate
Beta1
B.
Seph-RalA
Seph-RalB
Sepharose
Cell lysate
Beta1
C.
Seph-Beta1
Sepharose
Cell lysate
RalA
RalB

Figure 35

Fig. 35. Sepharose-CNBr-coupled RalA and RalB (or β1) interact *in vitro* with endogenous β1 (or RalA and RalB) in human platelets and canine sarcolemmal membranes. (A) Sepharose-CNBr-coupled RalA and RalB interact with endogenous β1 in human platelets. Outdated human platelets were prepared as described in Methods, lysed in Ral buffer and incubated with 50 μl Sepharose-CNBr-coupled RalA and RalB plus Sepharose-CNBr control beads. Bound proteins were boiled off the beads, solubilized in Laemmli buffer, separated by 13% SDS-PAGE, transferred to PVDF membranes and probed with anti-β1 Na^+/K^+-ATPase monoclonal antibody. Total platelet lysate was probed as a positive control. (B) Sepharose-CNBr-coupled RalA and RalB interact with endogenous β1 in canine sarcolemmal membranes. In a Sepharose-CNBr-fusion protein pull-down experiment described in Methods, 50 μl Sepharose-CNBr-coupled RalA and RalB plus Sepharose-CNBr control beads, were incubated with solubilized proteins from CSMs (100 μg). The beads were recovered, the proteins boiled off in Leammli's buffer and separated by 13% SDS-PAGE, and probed with anti-β1 monoclonal antibody. CSM supernatant (1 μg) was probed as a positive control. (C) Sepharose-CNBr-coupled β1 interacts with endogenous Ral in canine sarcolemmal membranes. In an identical Sepharose-CNBr-fusion protein pull-down experiment, 50 μl Sepharose-CNBr-coupled RalA (B, upper panel) and RalB (B, lower panel), plus Sepharose-CNBr control beads, were incubated with solubilized proteins from CSMs (100 μg). Bound proteins were separated and probed with anti-RalA (B, upper panel) and anti-RalB (B, lower panel) monoclonal antibodies. CSM supernatant (1 μg) was probed as a positive control. Experiments were repeated at least three times and gave identical results.

5.3.6 Recombinant *In Vitro* Transcribed/Translated [^{35}S]Met-labelled β1 Interacts with GST-RalA and GST-RalB *In Vitro*

Before performing the *in vitro* binding assay, it was first determined whether the pGADT7[β1] construct could be induced to translate [^{35}S]Met-labelled β1 using the TnT transcription/translation system in the presence of [^{35}S]methionine. Autoradiography determined that the pGADT7[β1] construct but not the empty plasmid produced [^{35}S]Met-labelled β1 (Fig. 36A). An [^{35}S]Met-labelled protein of about 35 kDa mass was translated, which conforms to unglycosylated HA-tagged β1 protein. This also verifies that the β1 cDNA was inserted in frame into the pGADT7 plasmid. To determine if β1 binds full length RalA and RalB *in vitro*, an *in vitro* binding assay was performed. Autoradiography (Fig. 36B) determined that both GST-RalA and GST-RalB, but not GST control beads, bound [^{35}S]Met-labelled β1. These results again prove that β1 interacts with Ral.

5.3.7 Ral Immunoprecipitates with β1 but not c-Src in HeLa Cells

To determine if Ral and β1 also interact in mammalian cell lines, co-immunoprecipitation experiments were performed in HeLa cells (Fig. 37). In cell lysates from quiescent HeLa cells, β1 antibody was used to precipitate RalA (Fig. 37A), and RalB antibody was used to precipitate β1 (Fig. 37B), with IgG2α and rabbit IgG, respectively, being used as negative controls. Results showed that β1 and RalB antibodies co-precipitated endogenous RalA and β1, respectively. Therefore, Ral and β1 form complexes in a mammalian cell line. The signal observed in the control IgG lane was due

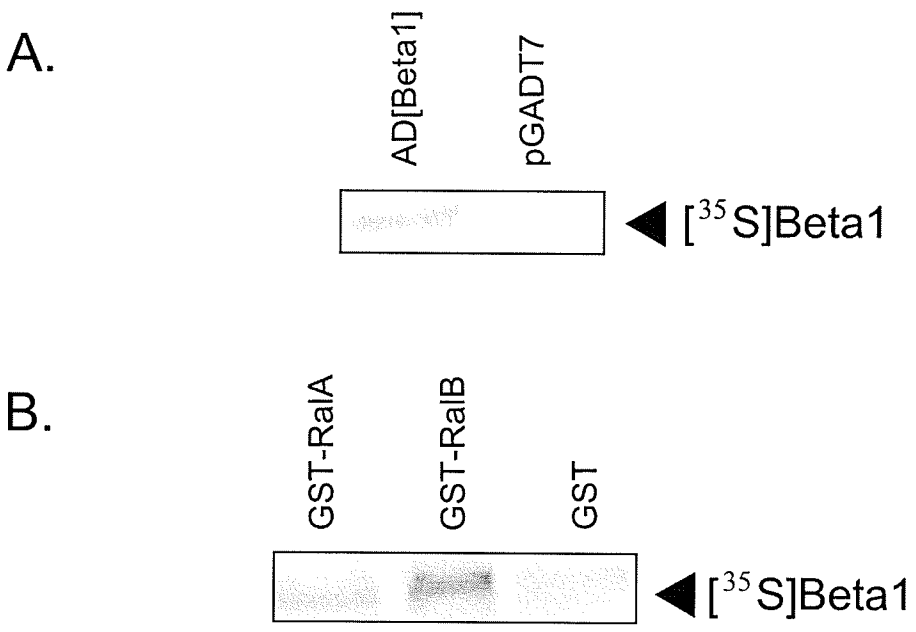


Figure 36

Fig. 36. Recombinant *in vitro*-transcribed/translated [^{35}S]Met-labelled β1 interacts with GST-RalA and GST-RalB *in vitro*. (A) β1 is translated from the pGADT7[β1] construct. An *in vitro* transcription/translation reaction was performed as described in Methods to determine if β1 was translated from the pGADT7[β1] template in the presence of [^{35}S]methionine. Any proteins translated were separated by 13% SDS-PAGE. The gel was vacuum-dried and autoradiography used to visualize radioactivity associated with the proteins. Experiments were repeated at least three times and gave identical results. (B) Recombinant *in vitro*-transcribed/translated [^{35}S]Met-labelled β1 interacts with GST-RalA and GST-RalB *in vitro*. In an *in vitro* binding assay, as described in Methods, 50 μl GSH-agarose-coupled GST-RalA or GST-RalB were incubated with *in vitro* transcribed/translated [^{35}S]Met-labelled β1. After one hour incubation, bound proteins were boiled off beads and separated by 13% SDS-PAGE. The gel was vacuum-dried and autoradiography used to visualize radioactivity associated with the proteins. Experiments were repeated at least three times and gave identical results.

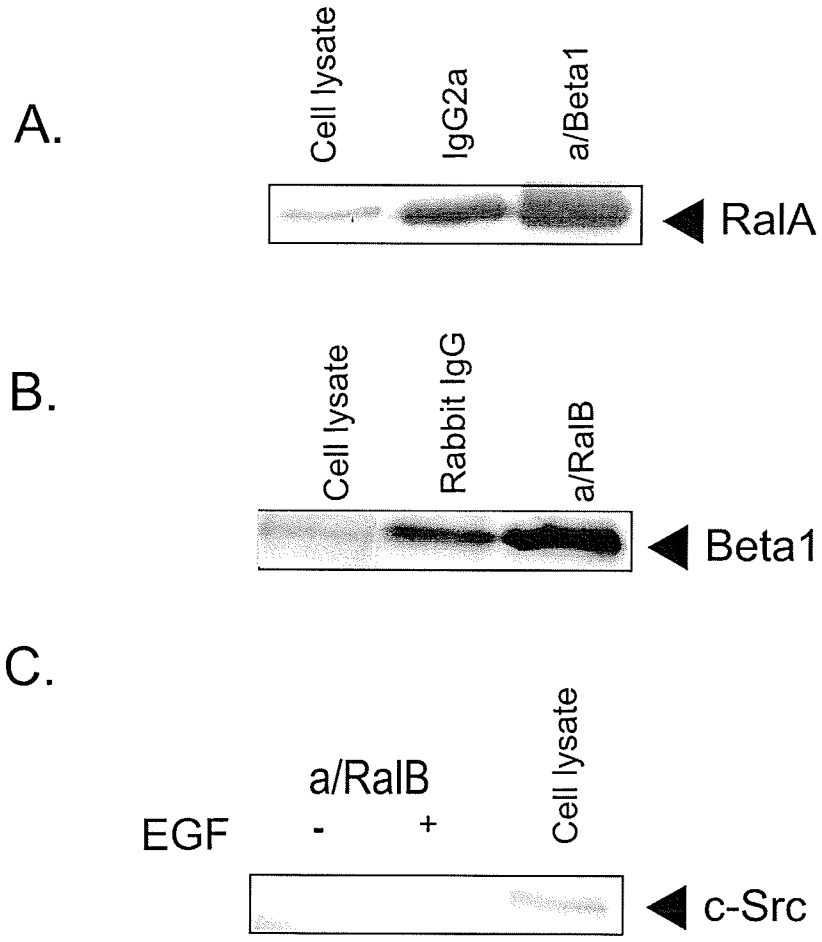

A.
Cell lysate
IgG2a
a/Beta1
RalA
B.
Cell lysate
Rabbit IgG
a/RalB
Beta1
C.
a/RalB
EGF
-
+
Cell lysate
c-Src

Figure 37

Fig. 37. Ral immunoprecipitates with β1 but not c-Src in HeLa cells. (A, B) Ral forms a complex with β1 in HeLa cells. Quiescent HeLa cells were lysed and sonicated in RIPA buffer plus protease inhibitors, and supernatants precleared with 20 μl protein A/G Plus-Agarose, and precipitated with 4 μg anti-β1 or IgG2a (A), or with anti-RalB or rabbit IgG (B) antibodies, respectively. Protein A/G Plus-Agarose was used to pull down immunoprecipitates which were dissolved in Laemmli's buffer. Proteins were boiled off the beads, separated by 13% SDS-PAGE, and transferred to PVDF membranes. The membranes were probed with anti-RalA (A) or anti-β1 (B) antibodies. Experiments were repeated at least three times and gave identical results. (C) Ral and c-Src do not co-immunoprecipitate in HeLa cells. HeLa cells, some of which were pre-treated for 5 minutes with 10 ng/ml recombinant EGF, were lysed and sonicated in RIPA buffer plus protease inhibitors, and supernatants precleared with 20 μl protein A/G Plus-Agarose, and precipitated with 4 μg anti-RalB or rabbit IgG (C) antibodies. Protein A/G Plus-Agarose was used to pull down immunoprecipitates which were dissolved in Laemmli's buffer. Proteins were boiled off the beads, separated by 13% SDS-PAGE, and transferred to PVDF membranes. The membranes were probed with anti-c-Src monoclonal antibody (C). Experiments were repeated at least three times and gave identical results.

to the fact that the heavy and light chains of IgG had almost identical mobility to that of β1 and Ral, respectively. As the protein bands could not be clearly separated, we based our conclusions on the difference in signal intensities. Because c-Src binds to the α subunit of Na^+/K^+-ATPase (Haas *et al.*, 2002) and we demonstrate Ral interacts with β1, we wanted to determine if Ral binds c-Src. Co-immunoprecipitation experiments in HeLa cells, using RalB to precipitate c-Src from cell lysate (Fig. 37C), showed that Ral and c-Src do not appear to form a complex in HeLa cells, whether the cells are stimulated or not with EGF.

5.3.8 Ouabain-induced Inhibition of Na^+/K^+-ATPase has a Minimal Effect on the Basal Activation State of Ral

Partial, non-toxic inhibition of Na^+/K^+-ATPase by cardiac glycosides such as ouabain activates signal transduction pathways which regulate the expression of cardiac hypertrophic genes (Kometiani *et al.*, 2000). Because Ral is involved in cardiac hypertrophy (Kawai *et al.*, 2002), and we have shown that Ral binds β1, we wished to determine whether Ral is involved in the ouabain-activated Na^+/K^+-ATPase/c-Src/Ras signal transduction pathways (Haas *et al.*. 2000, 2002). We first wanted to determine the effect of ouabain treatment on the activation state of Ral. Quiescent HeLa cells were treated with ouabain for 15 minutes (Fig. 38A) or 2 hours (Fig. 38B), and A7r5 cells were treated with ouabain for 15 minutes (Fig. 38C). Cell lysates were then incubated with GST-RRBD coupled to GSH-agarose beads to pull down activated Ral. Ouabain appears to cause a slight but reproducible increase in RalA (Fig. 38C, upper panel; 27% +/- 8% SD) or RalB (Fig. 38C, lower panel; 24% +/- 1% SD) activation over basal levels in

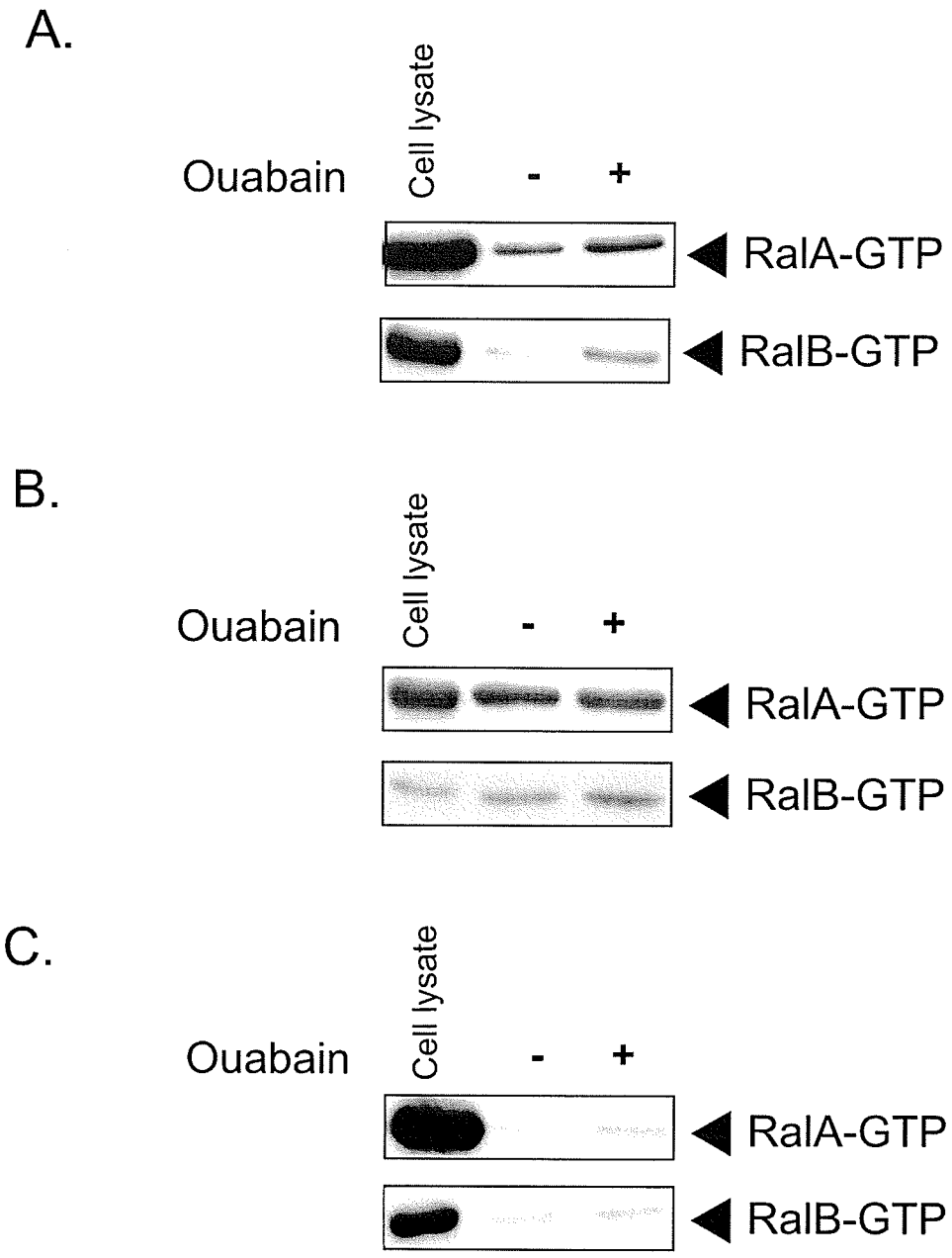

A.
Ouabain
Cell lysate
-
+
RalA-GTP
RalB-GTP
B.
Ouabain
Cell lysate
-
+
RalA-GTP
RalB-GTP
C.
Ouabain
Cell lysate
-
+
RalA-GTP
RalB-GTP

Figure 38

Fig. 38. Ouabain-induced inhibition of Na^+/K^+-ATPase has a minimal effect on the basal activation state of Ral. HeLa cells were treated with 1 μM ouabain for 15 (A) or 120 (B) minutes, and A7r5 cells were treated with 100 μM ouabain for 15 minutes (C). Cell lysates were incubated with 50 μl GST-RRBD coupled to GSH-agarose beads, bound proteins were boiled off the beads, separated by 13% SDS-PAGE, transferred to PVDF membrane and probed with anti-RalA or –RalB antibodies. Aliquots of lysates were taken before beads were added and probed with anti-β-actin or –Ras antibody to ensure equal loading (results not shown). Experiments were repeated at least three times and gave identical results.

quiescent A7r5 cells when treated for 15 minutes. However, quantification determined that a fifteen minute or two hour treatment of HeLa cells with ouabain resulted in no increase in RalA or RalB activation. These results indicate that short-term ouabain-induced inhibition of Na^+/K^+-ATPase minimally activates Ral in A7r5 cells, but does not activate Ral in HeLa cells.

5.3.9 Increased Intracellular Ca^{2+} Does Not Enhance Ral Activation in the Presence of Ouabain

To test whether the lack of full Ral activation in cells treated with ouabain is due to a lack of a surge in intracellular Ca^{2+} due to the absence of a Na^+/Ca^{2+}-exchanger in HeLa cells, cells were treated with thapsigargin or Ca^{2+} ionophore A23187 before treatment with ouabain, and RalGTP pulled down with GST-RRBD (Fig. 39). Increased intracellular Ca^{2+} had no additive effect on RalA activation in the presence of ouabain. The reason for this may be that ouabain inhibits Ca^{2+}-induced Ral activation, or that the extracellular Ca^{2+} levels in our assays were not high enough.

5.3.10 Inhibition of Na^+/K^+-ATPase Activity by Short-term Ouabain Treatment Inhibits EGF-induced Ral Activation in A7r5 but not HeLa Cells

Because an increase in intracellular Ca^{2+} does not appear to enhance the minimal ouabain-induced activation of Ral, we wished to determine the effect of ouabain treatment on Ral activation induced by EGF. EGF activates the c-Src/EGFR/STAT3 cascade through the Ras/RalGEF/Ral pathway (Goi *et al.*, 2000). A7r5 and HeLa cells were treated with EGF in the presence of ouabain, and active Ral detected with GST-

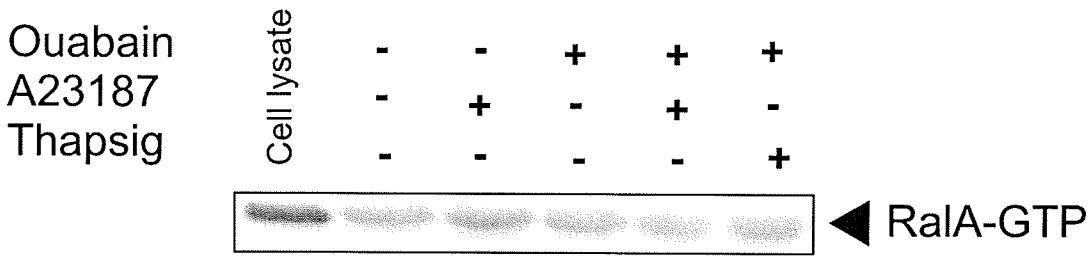


Figure 39

Fig. 39. Increased intracellular Ca^{2+} does not enhance Ral activation in the presence of ouabain. HeLa cells were treated with 2 μM thapsigargin or 10 μM A23187 in the presence of 0.2 mM $CaCl_2$. Thirty minutes later the cells were treated with 1 μM ouabain for 15 minutes. Cell lysates were incubated with 50 μl GST-RRBD coupled to GSH-agarose beads, bound proteins were boiled off the beads, separated by 13% SDS-PAGE, transferred to PVDF membrane and probed with anti-RalA antibody. Aliquots of lysates were taken before beads were added and probed with anti-β-actin or –Ras antibody to ensure equal loading (results not shown). Experiments were repeated at least three times and gave identical results.

RRBD (Fig. 40). In A7r5 cells, ouabain decreased RalA (Fig. 40A, upper panel) and RalB (Fig. 40A, lower panel) activation induced by EGF, while in HeLa cells, ouabain had no effect on EGF-induced RalA (Fig. 40B, upper panel) or RalB (Fig. 40B, lower panel) activation This suggests that in A7r5 but not HeLa cells, partial inhibition of Na^+/K^+-ATPase by ouabain inhibits Ral activation induced by EGF.

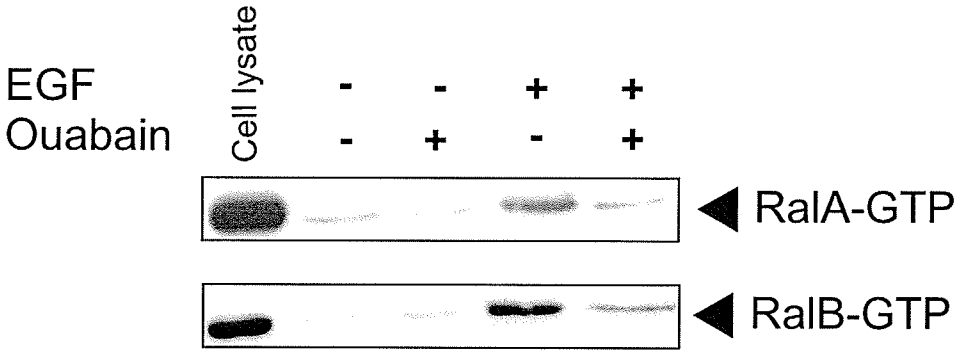
5.3.11 Inhibition of Na^+/K^+-ATPase Activity by Long-term Ouabain Treatment Inhibits Ral Activation by EGF

To determine whether longer term ouabain treatment (to activate early- and late-response and cardiac hypertrophic genes) affected Ral activation stimulated by EGF, A7r5 and HeLa cells were treated with ouabain for 24 hours followed by a 15 minute treatment with EGF. In A7r5 (Fig. 41A) and HeLa (Fig. 41B) cells, 24 hour ouabain treatment caused inhibition of both basal and EGF-induced RalA (Fig. 41A and B, upper panels) and RalB (Fig. 41A and B, upper panels) activation to below basal levels. Probing for β-actin showed equal protein was loaded and that long-term ouabain treatment did not cause a decrease in β-actin levels (results not shown). These results suggest that long-term ouabain-induced inhibition of Na^+/K^+-ATPase prevents activation of Ral by EGF, or alternatively, causes downregulation of Ral protein expression.

5.3.12 Inhibition of Na^+/K^+-ATPase Activity by Long-term Ouabain Treatment Inhibits Ral Protein Expression

To determine whether long-term ouabain treatment (24 hours) of cells affected Ral expression, A7r5 (Fig. 42A) and HeLa (Fig. 42B) cells were treated with ouabain for

A.


EGF
Ouabain
Cell lysate
- - + +
- + - +
RalA-GTP
RalB-GTP

B.

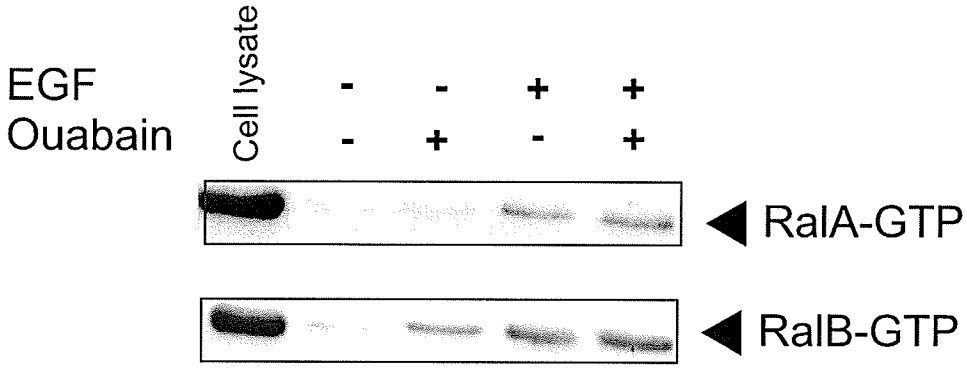

EGF
Ouabain
Cell lysate
- - + +
- + - +
RalA-GTP
RalB-GTP

Figure 40

Fig. 40. Inhibition of Na^+/K^+-ATPase activity by short-term ouabain treatment inhibits EGF-induced Ral activation in A7r5 but not HeLa cells. A7r5 (A) and HeLa (B) cells were treated with 100 and 1 μM ouabain, respectively, for 15 minutes, followed by treatment with 10 ng/ml recombinant EGF for 15 minutes. Cell lysates were incubated with 50 μl GST-RRBD coupled to GSH-agarose beads, bound proteins were boiled off the beads, separated by 13% SDS-PAGE, transferred to PVDF membrane and probed with anti-RalA and RalB antibodies. Aliquots of lysates were taken before beads were added and probed with anti-β-actin or –Ras antibody to ensure equal loading (results not shown). Experiments were repeated at least three times and gave identical results.

Fig. 41. Inhibition of Na^+/K^+-ATPase activity by long-term ouabain treatment inhibits Ral activation by EGF. A7r5 (A) and HeLa (B) cells were treated with 100 and 1 μM ouabain, respectively, for 24 hours, followed by treatment with 10 ng/ml recombinant EGF for 15 minutes. Cell lysates were incubated with 50 μl GST-RRBD coupled to GSH-agarose beads, bound proteins were boiled off the beads, separated by 13% SDS-PAGE, transferred to PVDF membrane and probed with anti-RalA and RalB antibodies. Aliquots of lysates were taken before beads were added and probed with anti-β-actin antibody to ensure equal loading (results not shown). Experiments were repeated at least three times and gave identical results.

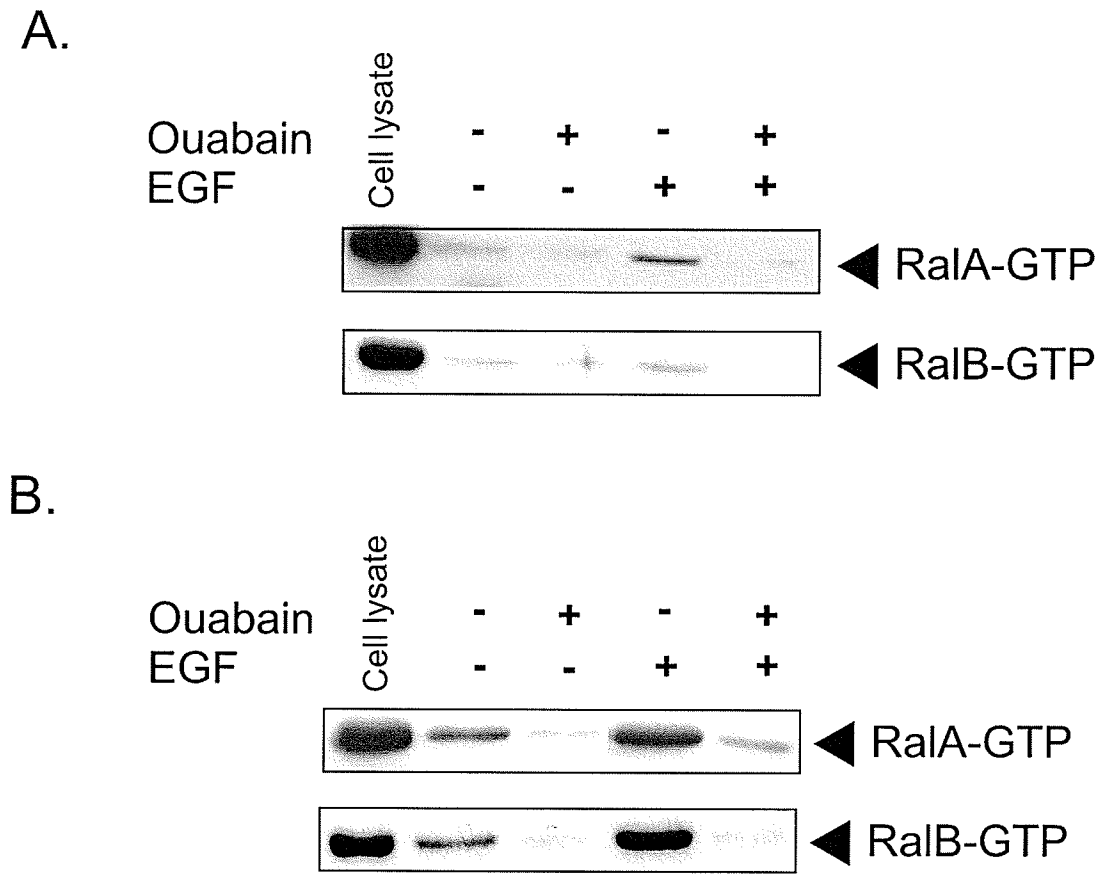

A.
Cell lysate
Ouabain
EGF
- + - +
- - + +
RalA-GTP
RalB-GTP
B.
Cell lysate
Ouabain
EGF
- + - +
- - + +
RalA-GTP
RalB-GTP

Figure 41

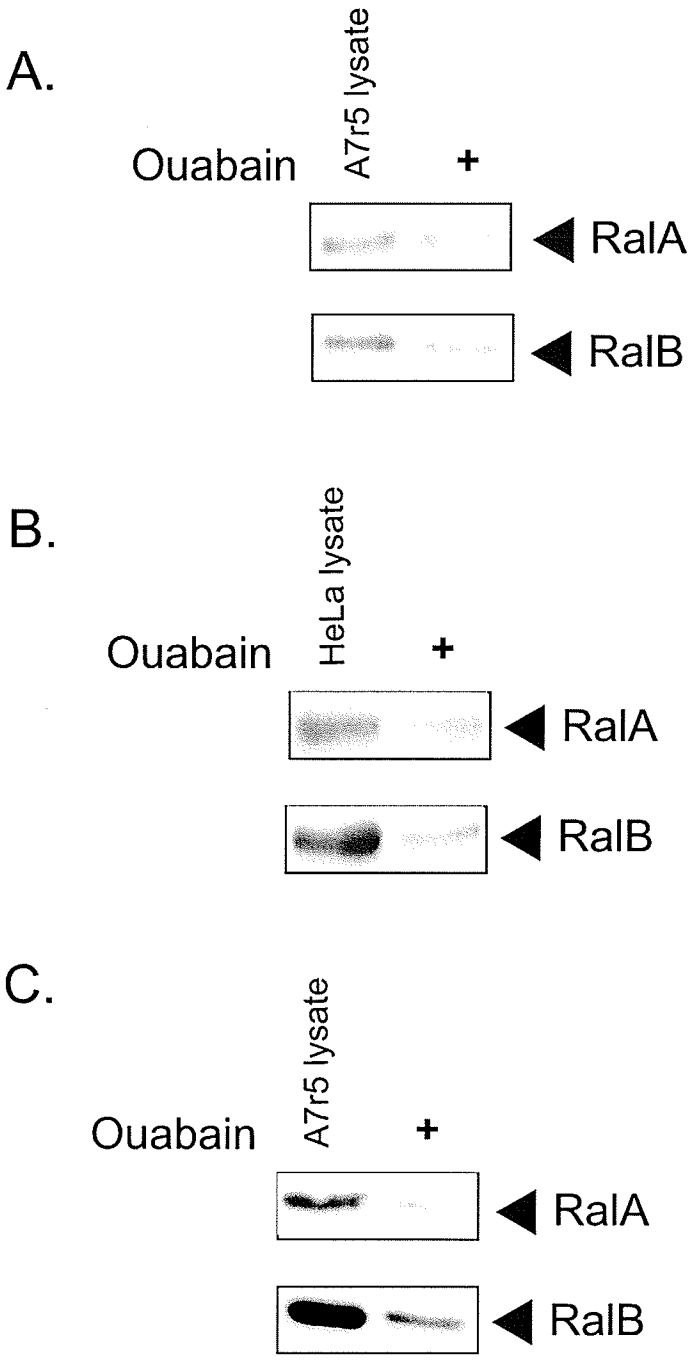

A.
A7r5 lysate
Ouabain
+
RalA
RalB
B.
HeLa lysate
Ouabain
+
RalA
RalB
C.
A7r5 lysate
Ouabain
+
RalA
RalB

Figure 42

Fig. 42. Inhibition of Na^+/K^+-ATPase activity by long-term ouabain treatment inhibits Ral protein expression. A7r5 (A, C) and HeLa (B) cells were treated with 100 (A), 20 (C) and 1 (B) μM ouabain, respectively, for 24 hours, followed by lysis in 2x laemmli buffer. Total proteins in cell lysates were separated by 13% SDS-PAGE, transferred to PVDF membrane and probed with anti-RalA (A, B, C, upper panels) or RalB (A, B, C, lower panels) antibodies. Lysates were also probed with anti-β-actin antibody to ensure equal loading (results not shown). Experiments were repeated at least three times and gave identical results.

24 hours and total Ral protein detected in cell lysates by western blot. Ouabain caused a profound decrease in RalA (Fig. 42A and B, lower panels) and RalB (Fig. 42A and B, lower panels) expression compared to controls. Probing for β-actin showed equal protein was loaded and that long-term ouabain treatment did not cause down-regulation of β-actin (data not shown). To further confirm that results were not due to a cytotoxic effect, A7r5 cells were also treated with 20% of the recommended dose of ouabain, and RalA (Fig. 42C, upper panel) and RalB (Fig. 42C, lower panel) protein expression was similarly suppressed. This suggests that inhibition of Na^+/K^+-ATPase by ouabain inhibits Ral expression, and conversely that a normal functioning pump maintains or up-regulates Ral expression.

5.3.13 Inhibition of Na^+/K^+-ATPase Activity by Long-term Ouabain Treatment Inhibits β1, Ras and CaM Protein Expression, an Effect that is Enhanced in A7r5 but not HeLa Cells by Ral

Ouabain and other hypertrophic stimuli cause increased β1 mRNA transcription in cardiac myocytes (Kometiani *et al.*, 2000). We wished to determine the effects of long-term ouabain treatment on β1 protein expression in A7r5 and HeLa cells. An 18 hour ouabain treatment caused marked reductions in the expression of β1 (Fig. 43A) and Ras (Fig. 43B) in A7r5 and HeLa (Fig. 43D, upper and lower panels, respectively) cells, as well as CaM in A7r5 cells (Fig. 43C). When cells were transfected with RalB(N28) six hours prior to ouabain treatment there was some sparing of the downward expression of both β1 and Ras in A7r5 (Fig. 43A and B) but not HeLa (Fig. 43D) cells. Probing for β-actin showed equal protein was loaded and that long-term ouabain treatment did not

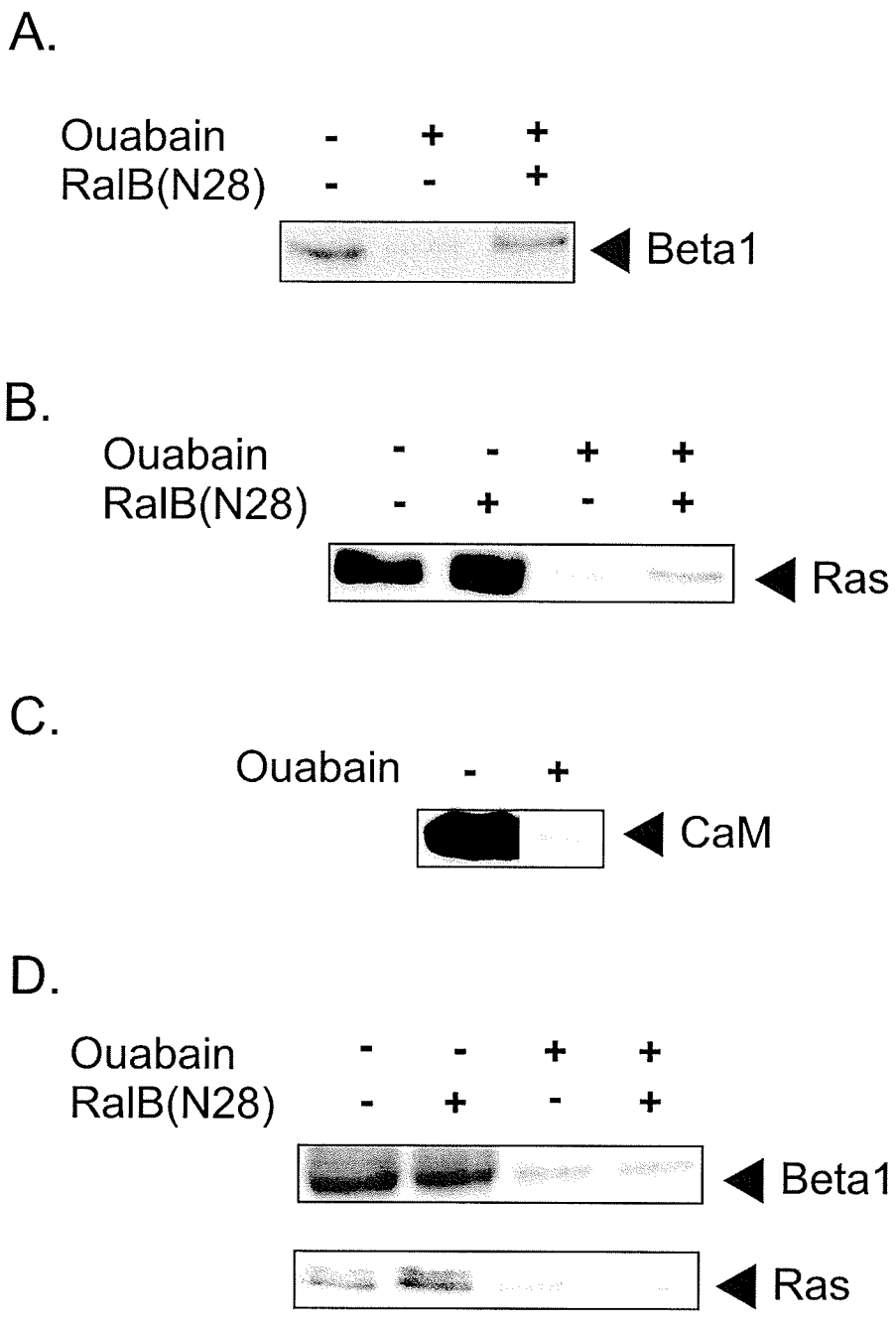

A.
Ouabain - + +
RalB(N28) - - +
Beta1
B.
Ouabain - - + +
RalB(N28) - + - +
Ras
C.
Ouabain - +
CaM
D.
Ouabain - - + +
RalB(N28) - + - +
Beta1
Ras

Figure 43

Fig. 43. Inhibition of Na^+/K^+-ATPase activity by long-term ouabain treatment inhibits β1-subunit, Ras and CaM protein expression, an effect that is enhanced in A7r5 but not HeLa cells by Ral. A7r5 (A-C) and HeLa (D) cells were treated with 100 and 1 μM ouabain, respectively, for 18 hours, followed by lysis in 2x laemmli buffer. Some cells were transfected with RalB(N28) 6 hours prior to ouabain treatment. Total proteins in cell lysates were separated by 13% SDS-PAGE, transferred to PVDF membrane and probed with anti-β1 (A and D [upper panel]), -Ras (B & D [lower panel]) or –CaM (C) antibodies. Lysates were also probed with anti-β-actin antibody to ensure equal loading (results not shown). Experiments were repeated at least three times and gave identical results.

cause down-regulation of β-actin (results not shown). These novel findings show that long-term non-toxic inhibition of Na^+/K^+-ATPase causes down-regulation of β1, as well as CaM and Ras, and that Ral enhances the ouabain-induced down-regulation of β1 and Ras protein expression in A7r5 but not HeLa cells. That β-actin levels were unaltered shows there was no ouabain-induced down-regulation of all proteins.

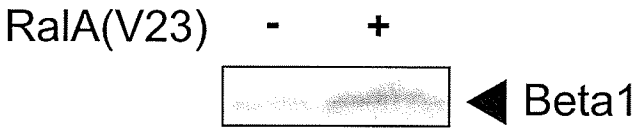
5.3.14 Constitutively Active RalA Upregulates β1 Protein Expression in A7r5 but not HeLa Cells

Because we show long-term ouabain-induced inhibition of Na^+/K^+-ATPase inhibits Ral protein expression, we wished to determine whether transfection of cells with constitutively active Ral mutants affected β1 expression. Western blotting of cell lysates showed that transfection of A7r5 cells with RalA(V23) (Fig. 44A) caused an upregulation of β1 expression after 24 hours. In contrast, transfection of HeLa cells with RalA(V23) (Fig. 44B, upper panel) or RalB(V23) (Fig. 44B, lower panel) had no effect on β1 expression after 24 hours. This suggests that increased Ral expression up-regulates β1 expression in A7r5 but not HeLa cells.

5.3.15 Calmodulin is Required for the Ouabain-induced Activation of c-Src in HeLa and A7r5 Cells

Ouabain's gene regulatory and hypertrophic effects are dependent on Ca^{2+}, CaM and PKC (Kometiani *et al.*, 2000). Because Ral is a CaM binding protein and is activated by Ca^{2+} and CaM, we wished to determine if CaM is required for activation of c-Src. Western blot with anti-c-Src p[Y^{418}] antibody showed that W7 inhibited ouabain-induced

A.



B.

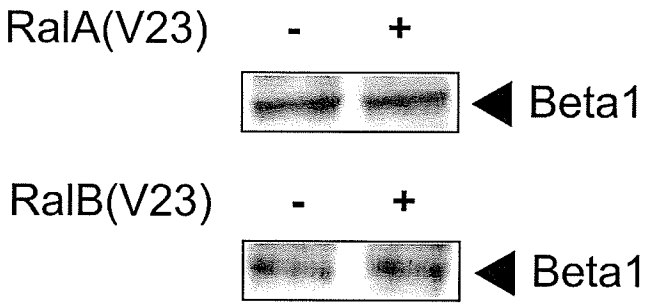


Figure 44

Fig. 44. Constitutively active RalA upregulates β1 protein expression in A7r5 but not HeLa cells. A7r5 cells (A) were transfected with RalA(V23), and HeLa cells (B) were transfected with RalA(V23) (upper panel) or RalB(V23) (lower panel). After 24 hours, the cells were lysed in 2x laemmli buffer, total proteins were separated by 13% SDS-PAGE, transferred to PVDF membrane and probed with anti-β1 antibody. Lysates were also probed with anti-β-actin antibody to ensure equal loading (results not shown). Experiments were repeated at least three times and gave identical results.

c-Src activation in both A7r5 (Fig. 45, upper panel) and HeLa (Fig. 45, lower panel) cells. This demonstrates that CaM is required for c-Src activation by ouabain.

5.3.16 Ral Inhibits Ouabain-induced Activation of c-Src in A7r5 and HeLa Cells

Ouabain treatment activates c-Src (Haas *et al.*, 2000), and induces c-Src to form a complex with the α subunit of Na^+/K^+-ATPase (Haas *et al.*, 2002). Because we show Ral binds directly to the $\beta 1$ subunit of Na^+/K^+-ATPase, and Ral is a CaM binding protein and is activated by Ca^{2+} and CaM, we wished to determine whether Ral is involved in the ouabain-induced activation of c-Src. Therefore, A7r5 (Fig. 46A, upper panel) and HeLa (Fig. 46A, lower panel) cells were transfected with RalB(N28) and then treated 24 hours later with ouabain for 5 minutes. Activated c-Src was determined by probing cell lysates with anti-c-Src p[Y^{418}] antibody. Results demonstrated that dominant-negative Ral caused an increase in ouabain-induced c-Src activation when compared to ouabain-treated untransfected cells. These results suggest that endogenous Ral inhibits c-Src activation by ouabain.

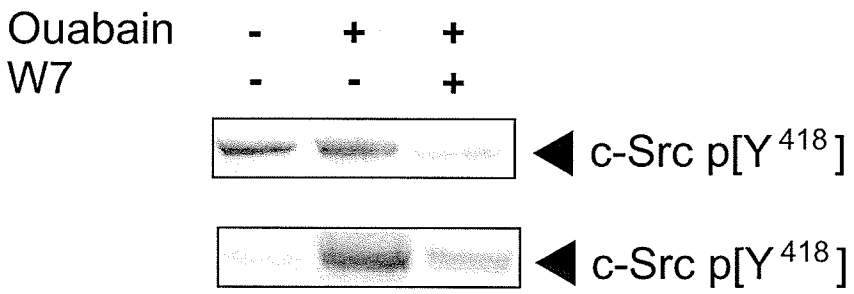


Figure 45

Fig. 45. Calmodulin is required for the ouabain-induced activation of c-Src. A7r5 (upper panel) and HeLa (lower panel) cells were treated with 50 μM W7 for 30 minutes, followed by a 5 minute treatment with 100 and 1 μM ouabain, respectively. Cells were lysed in modified RIPA buffer, total proteins were separated by 13% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-Src p[Y^{418}] antibody. Membranes were also probed with anti-Ras antibody to ensure equal loading (results not shown). Experiments were repeated at least three times and gave identical results.

Fig. 46. Ral inhibits ouabain-induced activation of c-Src p[Y^{418}] in A7r5 and HeLa cells. A7r5 (upper panel) and HeLa (lower panel) cells were transfected with RalB(N28) and treated 24 hours later with 100 and 1 μM ouabain, respectively, for 5 minutes. Cells were lysed in modified RIPA buffer, total proteins were separated by 13% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-Src p[Y^{418}] antibody. Lysates were also probed for β-actin to ensure equal loading (results not shown). Experiments were repeated at least three times and gave identical results.

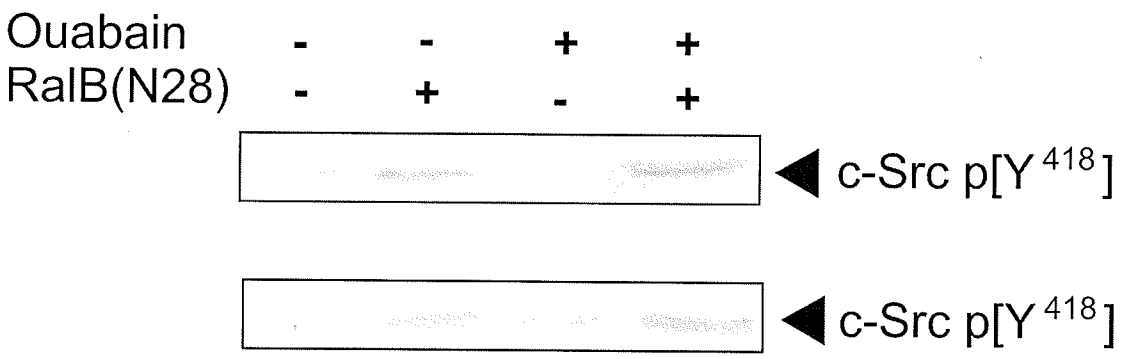


Figure 46

6. DISCUSSION

6.1 Calmoduluin Binds RalA and RalB and is Required for their Activation in Human Platelets

It has been previously reported that elevated levels of Ca^{2+} activate RalA (Hofer *et al.*, 1994, 1998; Urano *et al.*, 1996; Wolthuis *et al.*, 1998b; Zwartkruis *et al.*, 1998; M'Rabet *et al.*, 1999; Wang and Roufogalis, 1999; Park, 2001), and that CaM binds RalA *in vitro* by direct binding of CaM to the C-terminus of RalA (Wang *et al.* 1997; Wang and Roufogalis 1999). This suggests that RalA is associated with Ca^{2+}/CaM-dependent intracellular signalling pathways (Wang *et al.* 1997). As well, platelet agonists (e.g. platelet activating factor, thromboxane A_2, and α-thrombin) activate RalA in platelets in a Ca^{2+}-dependent manner, suggesting that RalA activation is mediated by a common signalling event that may involve Ca^{2+} (Wolthuis *et al.*, 1998b). We propose that CaM is an essential component in this process. We have shown, for the first time, by co-immunoprecipitation in human platelets and yeast two-hybrid assays, that RalB binds CaM *in vitro*, and that both RalA and RalB interact specifically and directly with CaM *in vivo* in a eukaryotic system. These results were confirmed and shown to be Ca^{2+}-dependent by *in vitro* binding assays. The co-immunoprecipitation results show that active (thrombin-stimulated) and inactive (Clough *et al.*, 2002) endogenous Ral form complexes with endogenous CaM in platelets. Results from the Y2H assays importantly demonstrate that Ral interacts directly with CaM in a eukaryotic system, and appear to show that RalA binds CaM more readily than RalB. This was based solely on the qualitative finding that positive blue RalA/CaM colonies formed more quickly and had a more intense blue colouration than RalB/CaM colonies, even though western analysis showed similar protein expression levels. We speculate that this differential binding of

CaM by the Ral proteins may determine functional differences between RalA and RalB, such as related to Ca^{2+}/CaM signalling pathways. There is evidence the Ral proteins have different functions. RalA and RalB have differential functions in the regulation of transformed tissue culture cell growth. RalA is required for anchorage-independent proliferation of transformed cells, while RalB is required to prevent transformed cells from initiating apoptosis (Chien and White, 2003). The fact that in platelets RalA is exclusively membrane-bound, while RalB is found in both membranes and cytosol (Jilkina and Bhullar, 1996), also suggests RalA and RalB may have differential functions. The Ral proteins being involved in separate intracellular pathways does not preclude some of their separate pathways from uniting, via CaM, into a common pathway. Being highly identical, they are also likely to have many of the same or overlapping functions that may involve Ca^{2+}/CaM.

The regulation of Ral function by CaM appears to be complex. It has previously been reported (Wang *et al.*, 1997) that the CaM BD in RalA is at the C-terminus (Fig. 12). Because the two Ral proteins differ mainly at the C-terminus (Fig. 1), it was assumed RalB does not bind CaM. However, we have shown RalB also binds CaM, and wished to determine if there was a CaM BD in another region of RalB and perhaps RalA. The sequences of RalA and RalB were analysed by the CaM target data base (http:/calcium.oci.utoronto.ca/). This revealed the presence of a potential N-terminal CaM BD in RalB (average propensity for α-helix formation, 1.052) (Fig. 12). Examination of RalA showed a second high scoring putative N-terminal CaM BD (average propensity 0.957) when the C-terminal thirty residues containing the predicted CaM BD (Wang *et al.*, 1997) were removed to allow for a pattern search to identify any additional potential

CaM BDs (Fig. 12). The putative N-terminal CaM BD of RalB lies immediately upstream of the nucleoside phosphate-binding region, while that of RalA overlaps the nucleoside phosphate-binding region (Figs. 1 and 12). RalA and RalB have almost identical values for various parameters obtained from the analysis of the predicted N-terminal CaM binding sites. The mean hydrophobicity (-0.125, RalA and 0.135, RalB) and hydrophobic moments (0.302, RalA and 0.628, RalB) for the N-terminal regions of both proteins are within the range of values of most CaM BDs (Wang *et al.*, 1997). The major difference between the putative N-terminal CaM BDs and the predicted C-terminal CaM BD of RalA is that the latter forms a hydrophilic alpha-helical wheel (Wang *et al.*, 1997), while the N-terminal regions form hydrophobic alpha-helical wheels. The interaction of CaM with its target proteins is predominantly hydrophobic (Wang *et al.*, 1997). Both N- and C-terminal regions carry a net positive charge (+3 for N-terminal and +9 for C-terminal), as binding of CaM with target proteins also involves strong electrostatic interactions (Wang *et al.*, 1997).

Therefore, the existence of more than one CaM BD being present in the Ral proteins was examined. To test what effect on CaM binding removal of the C-terminal regions of the Ral proteins would have, the appropriate regions were deleted from RalA and RalB and the subsequent truncated proteins tested for interaction with CaM. Results demonstrated that $RalA_{1\text{-}549}$, which lacks the predicted CaM BD (Wang *et al.*, 1997), and $RalB_{1\text{-}482}$, which lacks the region equivalent to that of the CaM BD of RalA, interacted specifically and directly with CaM in the Y2H and *in vitro* binding assays. The *in vitro* results showed the interaction between full length Ral and CaM to be Ca^{2+}-dependent, and that the $RalB_{1\text{-}482}$ construct bound CaM much more readily than full length RalB.

This suggests that the C-terminal 40 amino acids of RalB normally inhibit, in some way, the RalB-CaM interaction, which may explain the *in vivo* findings that CaM interacts with RalB less readily than with RalA. The existence of an inhibitory domain has a precedent in that H-Ras has also been determined to have a CaM binding inhibitory domain (Villalonga *et al.*, 2001). Results with $RalB_{1-482}$ also showed that binding in the absence of Ca^{2+} was equal to or greater than the binding with CaM in the presence of Ca^{2+}. Therefore, it is possible that Ca^{2+} may, in certain circumstances, partially inhibit the binding of CaM to full length RalB. Our results, together with the fact that the double N- and C-terminally truncated RalB construct failed to bind CaM *in vitro* and very weakly *in vivo*, indicate that RalA and RalB have an N-terminal and a C-terminal CaM BD, or alternatively, that both regions are necessary for binding. The Ca^{2+}-independence of CaM binding to the C-terminally truncated RalB product, suggests that any remaining CaM BD (e.g. N-terminal) is Ca^{2+}-independent. Apocalmodulin (apoCaM) is a form of CaM that preferentially interacts with CaMBPs in the absence of Ca^{2+} (Jurado *et al.*, 1999). However, conditions that we are calling Ca^{2+}-independent may in fact be conditions of extremely low Ca^{2+} concentrations that can occur in the presence of chelators (Jurado *et al.*, 1999) or in resting cells.

In support of our findings that there are more than one CaM BDs in Ral, constructs $RalA_{1-264}$ and $RalB_{1-316}$, both containing the approximate N-terminal half of each protein, strongly bound CaM *in vitro* in the presence of either Ca^{2+} or EDTA. In contrast, constructs $RalA_{265-621}$ and $RalB_{317-621}$, both containing the approximate C-terminal half of each protein, strongly bound CaM *in vitro* in the presence of Ca^{2+} only. These results suggests that the C-terminal halves of RalA and RalB contain a Ca^{2+}-

dependent CaM BD motif, whereas the N-terminal halves contain a Ca^{2+}-independent CaM BD. This is supported by the *in vitro* binding assay results with the N- and C-terminally truncated RalB construct ($RalB_{100-482}$) which failed to bind CaM. Results with the N-terminal half constructs suggest that Ca^{2+} may partially inhibit the N-terminal Ca^{2+}-independent CaM BDs (compare lanes 1 and 3, Fig. 14, upper and lower panels) in RalA and RalB. In the cell, the avidity of the N-terminal CaM BD may vary with $[Ca^{2+}]_i$. Although removal of large regions of the Ral proteins may have altered the normal 3-D structure of the remaining portions and therefore changed their CaM binding properties, the results do suggest that RalA and RalB have two CaM interacting regions that vary in their Ca^{2+} dependence. We have therefore shown that there is an additional CaM BD in RalA from that described in the literature (Wang *et al.*, 1997), and at least two CaM BDs in RalB.

We speculate that in the cell, both RalA and RalB strongly bind CaM, with RalA having higher affinity for CaM than RalB. This differential binding of CaM may be regulated by the C-terminal inhibitory region of RalB that contains the Ca^{2+}-dependent CaM BD. Possible mechanisms controlling CaM binding at the C-terminus of RalB include a molecule binding to the CaM BD that inhibits CaM binding, or the putative CaM/RalB-binding inhibitory domain is masked or inhibited by another molecule, or a change in conformation in this region affects CaM binding. Because this C-terminal region contains an apparent Ca^{2+}-dependent CaM BD, the suggested molecular or conformational regulation may be controlled by Ca^{2+}. In one model, we propose that at low Ca^{2+} levels the inhibitory molecule remains bound to the CaM BD, but is released at higher intracellular Ca^{2+} levels to allow CaM to bind the C-terminal Ca^{2+}-dependent CaM

BD of RalB. In another model, the inhibitory domain is masked at high intracellular Ca^{2+} concentrations, and is open and fully active at low Ca^{2+} levels. Therefore, at higher intracellular Ca^{2+} levels a molecule binds to and masks the inhibitory domain, which allows CaM to bind, thus activating Ral. At lower Ca^{2+} concentrations, the masking molecule may dissociate from the inhibitory domain, thus disrupting CaM's interaction with RalB at this site. In the third model, the conformation of the CaM BD region is changed at different Ca^{2+} concentrations, which inhibits or favours CaM binding to RalB. This regulatory mechanism may not be active on RalA, thus accounting for the differential binding of RalA and RalB to CaM. In addition, optimal binding of CaM to RalA and RalB may require both Ca^{2+}-dependent and Ca^{2+}-independent CaM BDs being occupied.

Because Ca^{2+} activates RalA and RalB, the sensitivity of the Ral proteins to Ca^{2+} may have potential physiological significance, and there may be a direct functional regulation of RalA (Hofer *et al.*, 1994, 1998; Urano *et al.*, 1996; Wolthuis *et al.*, 1998b; Zwartkruis *et al.*, 1998; M'Rabet *et al.*, 1999; Wang and Roufogalis, 1999; Park, 2001) and, we suggest, RalB by Ca^{2+}/CaM in the physiological range. We propose the existence of both Ca^{2+}-dependent and Ca^{2+}-independent CaM BDs allows Ral to bind CaM over a wide range of physiological Ca^{2+} concentrations. This suggests such interactions are essential for cell function, and that Ral and CaM respond in concert to any change in physiological Ca^{2+} concentration. If the avidity of the N-terminal CaM BD does vary with Ca^{2+} concentration, it would allow fine tuning of Ral activation by CaM. CaM may be a potential effector for Ral GTPase activation and serve as a molecular switch in response to changes in intracellular Ca^{2+} concentration over the physiological range. It

would serve this role as an additional player in conjunction with RalGAP and RalGEF regulatory factors (Wang *et al.*, 1997).

It is important to understand the molecular mechanisms involved in Ral activation. It has been proposed that phosphorylation by Ca^{2+}-dependent kinases may regulate the activity of RalGEFs and RalGAPs, or that there may be Ca^{2+}-dependent RalGEFs (Wolthuis *et al.*, 1998b). Ral activation could also occur via Ras family members acting as upstream activators of RalGEFs (Feig *et al.*, 1996), such as by Ca^{2+}-activated Rap1 (Suzuki *et al.*, 2000). Increased Ca^{2+} can activate RalA and RalB via enhancing binding to GTP and inhibition of GDP binding to RalA (Park, 2001).

Our work with human platelets has shown that thrombin induces the activation of both RalA and RalB in a CaM-dependent manner. This would suggest that the Ca^{2+}-dependent platelet agonist-induced (Wolthuis *et al.*, 1998b) and Ca^{2+}-induced (Franke *et al.*, 1997; Wolthuis *et al.*, 1998b) activation of RalA reported previously, may in fact have been both CaM- and Ca^{2+}-dependent. It is likely that Ral is activated by many of the CaM-regulated intracellular pathways by forming a complex with CaM and its associated proteins. Several studies propose Ca^{2+} is involved in Ral activation by various mechanisms (Feig *et al.*, 1996, Wolthuis *et al.*, 1998b; Park, 2001). We speculate that CaM may be required for some, if not all, of these Ca^{2+}-dependent regulatory pathways. Ca^{2+}/CaM induces GTP binding to RalA in human erythrocyte plasma membrane (Wang *et al.*, 1997; Hofer *et al.*, 1998, Wang and Roufogalis, 1999), and the cycling of RalA in synaptic vesicle membranes by CaM is Ca^{2+}-dependent (Park *et al.*, 1999; Park, 2001). Because Ca^{2+}-activated CaM slightly decreased the GTP level through stimulation of RalA's GTPase activity, Ca^{2+} may stimulate GTP/GDP turnover on RalA (Park, 2001)

and RalB. Our results demonstrating the thrombin-induced, CaM-dependent activation of RalA and RalB in platelets, plus results cited from the literature, suggest that Ca^{2+}/CaM activate both RalA and RalB, and that the signalling pathways of Ca^{2+}/CaM and RalA and RalB are directly linked.

6.1.1 Model Depicting CaM-dependent, Thrombin-induced Activation of Ral in Human Platelets

In human platelets, thrombin binds to its receptors (protease-activated receptors 1 and 4, PAR-1 and PAR-4) and activates the heterotrimeric G-proteins G_q, G_{i2} and $G_{12/13}$ (Offermanns *et al.*, 2000) (Fig. 47). The α subunit of G_q interacts with and stimulates PLCβ to cleave PIP_2 into IP_3 and DAG. IP_3 induces an increase in intracellular Ca^{2+} levels which activates CaM and subsequently Ral. In addition to the pathways described in this model, thrombin stimulates Ca^{2+} release from internal stores in complementary but independent c-Src- and Ras-mediated pathways (Rosado *et al.*, 2000). Ras and c-Src act through reorganization of the actin cytoskeleton (Rosado *et al.*, 2000), and therefore may occur via thrombin-stimulated G_{i2} and $G_{12/13}$. Once activated by thrombin, Ral binds to the exocyst complex to stimulate platelet granule secretion.

6.2 Regulation of Ral Activation in Mammalian Cells

We next wantd to study mechanisms of Ral activation in proliferating cells. A major mechanism of Ral activation in mammalian cells is via growth factors such as EGF. Ral is activated by EGF via Ras and Ras-dependent RalGEFs. Ral activates c-Src which subsequently leads to activation of STAT3 and cortactin, but not the Ras/Raf/ERK

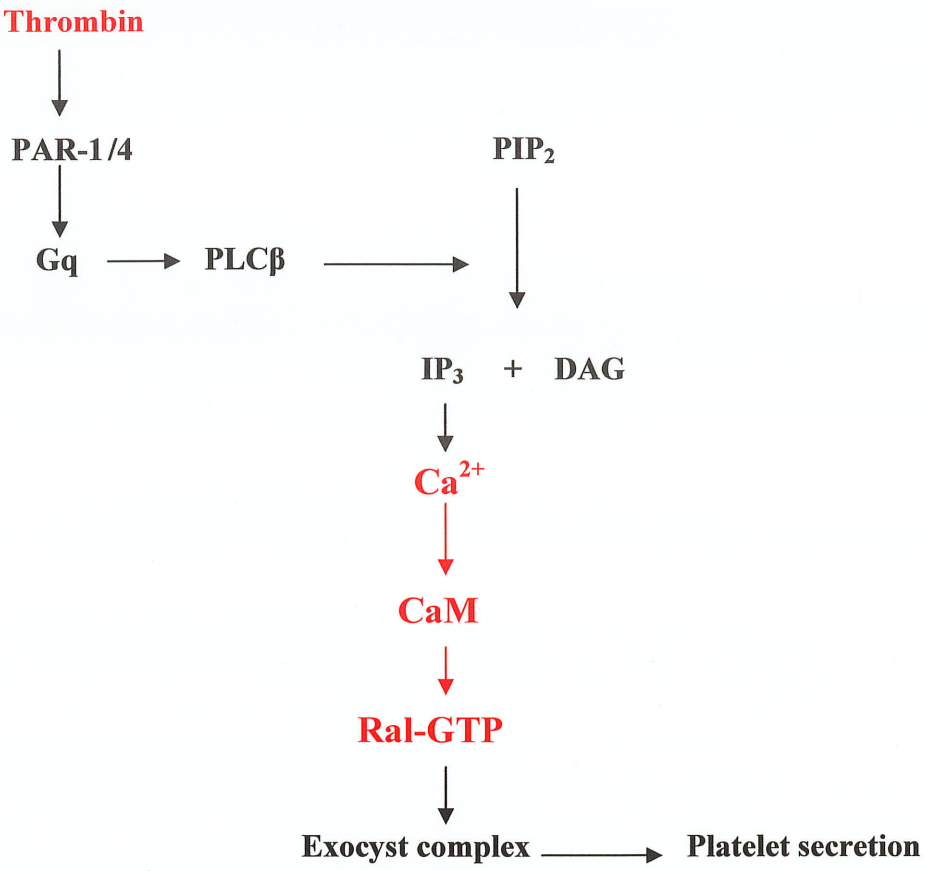


Fig. 47. Model depicting CaM-dependent, thrombin-induced activation of Ral in human platelets. In platelets, thrombin via its GPCR induces the activation of multiple signal transduction pathways, including CaM-regulated pathways. In one pathway, activated $G_{\alpha q}$ stimulates PLC to cause an IP_3-mediated increase in intracellular Ca^{2+}. The increased Ca^{2+} activates CaM which in turn activates Ral. Active Ral binds the exocyst complex to mediate secretion of granule contents from platelets.

cascade (Goi et al., 2000). The role of other proteins in the activation of Ral by EGF is not clear. We have shown above that thrombin activates Ral in platelets in a Ca^{2+}/CaM-dependent manner (Clough *et al.*, 2002), and we show here in A7r5 and MCF7 cells that CaM is also required for Ral activation by EGF, as well as basal activation. By contrast, in HeLa cells EGF-induced RalA and RalB activation is CaM-independent. However, Ca^{2+} is required in both A7r5 and HeLa cells tested, and is likely to be necessary for Ral activation by EGF in all cells. We postulate that in HeLa cells, EGF-induced Ral activation may involve an activated or upregulated RalGEF that acts independently of CaM-regulated pathways.

Because PLCγ binds to the EGF-stimulated EGFR, we propose that one source of Ca^{2+} that activates Ral is the EGF-induced rise in intracellular Ca^{2+} that occurs through the PLCγ/phosphoinositide signalling pathway (Schlessinger, 2000; Prenzel *et al.*, 2001). This allows downstream effects of EGFR stimulation to be mediated by Ca^{2+}-dependent signalling pathways. However, we show that the requirement for PLC is cell type-specific. PLC activation by EGF is required in EGF-induced activation of Ral in A7r5 cells. By contrast, in MCF7 and HeLa cells the EGF-induced activation of RalB but not RalA occurs via a PLC-dependent manner. Therefore, RalA and RalB may be differentially regulated in MCF7 and HeLa cells. This is only the second report of differential regulation of RalA and RalB. RalA, but not RalB, is activated by the IL-6/Janus kinase/STAT3/RalGDS pathway in M1 mouse myeloid leukaemia cells (Senga *et al.*, 2001). We postulate that in MCF7 and HeLa cells, RalA may require very low levels of intracellular Ca^{2+} for activation, while RalB requires a second spike in intracellular Ca^{2+} levels to be fully activated. RalA may be activated first, accompanied by low grade

RalB activation, which is followed by a secondary Ca^{2+} spike-induced full activation of RalB. We have determined above that the region containing the C-terminal Ca^{2+}-dependent CaM BD in RalB is relatively inhibitory to CaM binding (Clough *et al.*, 2002). This finding has a precedent in that H-Ras has also been determined to have a CaM binding inhibitory domain (Villalonga *et al.*, 2001). It is possible that in certain cells the second Ca^{2+} spike is necessary to overcome the inhibitory effects of this region to CaM binding, allowing full Ca^{2+}/CaM-induced activation of RalB.

Optimal PLC-mediated Ral activation depends on the increased intracellular Ca^{2+} produced by the IP_3 branch, and/or the activated PKC produced by the DAG branch of the PLC/phosphoinositide pathway. As we found for PLC, the requirement for PKC in EGF-induced Ral activation is also cell type-specific. We find that the EGF-induced activation of Ral depends on PKC, primarily the Ca^{2+}-independent, DAG-dependent δ isoform, in A7r5 but not HeLa cells. These findings appear to contradict those of Rusanescu *et al.*, (2001) in PC-12 and COS-7 cells. They reported that NGF- and EGF-induced activation of Ral in PC12 and COS-7 cells, respectively, is suppressed by PDA-stimulated PKC activity, an effect that is restored in PC12 cells by treatment with the general PKC inhibitor, GF109203X. Their results indicate that receptor activation of PKC preferentially suppresses Ras-induced Ral activation in PC-12 and COS-7 cells (Rusanescu *et al.*, 2001), while our results show PKC activates Ral in response to EGF treatment in A7r5 cells, but has no stimulatory or inhibitory effect in HeLa cells. The results in HeLa cells suggest again that in these cells Ral is not regulated by Ras, or that the DAG-independent atypical isoforms of PKC (ζ, λ) are involved. Therefore, we propose that PKCδ, PLC, Ca^{2+}, CaM, and growth factors act together to activate Ral in

A7r5 cells, while EGF-induced Ral activation in HeLa cells occurs primarily in a PKC-independent or atypical PKC-dependent and/or Ras-independent manner. Our results and the literature also suggest Ras and Ral are differentially activated by PKC. The discrepancies in our results and those of Rusanescu *et al.*, (2001) may be due to separate pools or isoforms of PKC being activated by the different stimuli, or due to the different cell types used.

It has been shown in PC-12 cells that that Ral is initially activated for about 10 minutes by NGF, but that by 30 minutes of stimulation this Ral activation is inhibited (Goi *et al.*, 1999) in a PKC-dependent manner (Rusanescu *et al.*, 2001). By contrast, we show in quiescent A7r5 and HeLa cells that PMA-stimulated PKC activates RalA and RalB in a Ca^{2+}/CaM- and time-dependent manner over a 30 minute period, and that the δ isoform of PKC at least is involved. These results suggest that phorbol ester-stimulated PKC is stimulatory, not inhibitory, to Ral activation in HeLa and A7r5 cells, and that EGF and PMA activate different isoforms of PKC. For example, PMA activates a PKC isoform in HeLa cells (e.g. PKCδ) that is required for Ral activation. However, PKCδ is not involved in EGF-induced Ral activation in HeLa cells.

The results with HeLa cells also suggest that EGF-induced Ral activation can occur via Ras-independent pathways. Therefore, we examined in more detail the activation of Ral by RalGEFs. Ral can be activated by Ras-dependent and Ras-independent pathways (Bos, 1998). Ras-independent pathways include Ca^{2+}/CaM-dependent (Hofer *et al.*, 1994, 1998; Urano *et al.*, 1996; Wolthuis *et al.*, 1998b; Zwartkruis *et al.*, 1998; M'Rabet *et al.*, 1999; Wang and Roufogalis, 1999; Park, 2001; Clough *et al.*, 2002) and Ras-independent RalGEF (Rebhun *et al.*, 2000; Gotoh *et al.*,

2000) pathways. Cell transfection experiments with dominant-negative RalB(N28) and over-expressed $p130^{Cas}$ demonstrated that the use of different RalGEFs varies between cell types. The Ras/RalGEF pathway is essential for both basal and EGF-induced RalA activation in A7r5 but not HeLa cells, and this effect is CaM-dependent. Dominant-negative Ral actually increases basal RalA activation in HeLa cells, suggesting Ras-dependent RalGEFs do not operate to activate RalA. There was no additive effect on RalA activation in HeLa cells transfected with RalB(N28) and treated with EGF, suggesting Ras-dependent RalGEFs are not prime candidates in inducing RalA activation in HeLa cells. By contrast, the Ras-independent RalGEF, BCAR3/AND-34 is required for basal and EGF-induced activation of RalA in HeLa but not A7r5 cells, and acts independently of CaM. It is possible that BCAR3 is the primary RalGEF activated by EGF in HeLa cells. We demonstrated that $p130^{Cas}$ caused almost total inhibition of Ral activation in HeLa cells. In addition, $p130^{Cas}$ did not inhibit another Ras-independent RalGEF, RalGPS1B, and there was no difference in RalA activation in HeLa cells treated with EGF and those treated with EGF and transfected with RalGPS1B. Therefore, we propose that EGF-induced activation of Ral in A7r5 cells occurs through Ras/RalGEF, and is enhanced by Ca^{2+}-, CaM-, PLCγ- and PKCδ-mediated pathways. In A7r5 cells, BCAR3/AND-34 may be a weak RalGEF that plays an insignificant role when compared to the Ras-dependent RalGEFs. In HeLa cells, EGF-induced activation of Ral occurs primarily through BCAR3/AND-34, and is enhanced by Ca^{2+}- and, for RalB only, PLCγ-mediated pathways.

Both $p130^{Cas}$ and AND-34 appear to activate common signalling pathways when over-expressed (e.g. c-Src pathways) (Hakak and Martin, 1999; Goi *et al.*, 2000), and

both function in a common signalling pathway to regulate hormone-dependent proliferation of breast cancer cells (Gotoh *et al.*, 2000). They are also implicated in the resistance of breast cancer cells to the anti-estrogen tamoxifen (van Agthoven *et al.*, 1998; Brinkman *et al.*, 2000; Van der Flier *et al.*, 2000), an effect that may depend on Ral, Rap1 and R-Ras (Gotoh *et al.*, 2000). We are proposing a molecular mechanism that may explain, in part, how this effect works. Through $p130^{Cas}$ transfection experiments in MCF7 cells, we show that over-expression of $p130^{Cas}$ enhances basal and EGF-induced RalA activation and, we speculate, Ral-dependent pathways (e.g. c-Src). Although BCAR1/$p130^{Cas}$ inhibits BCAR3/AND-34, Ral is activated here by $p130^{Cas}$ because estrogen-dependent MCF7 cells do not express BCAR3 (Cai *et al.*, 2003). This may occur by BCAR1/$p130^{Cas}$ activating other RalGEFs in MCF7 cells. Over-expression of $p130^{Cas}$ is known to activate c-Src (Burnham *et al.*, 2000), and our results suggest that BCAR1/$p130^{Cas}$-induced activation of c-Src is mediated via Ral. We also determined that when both RalGPS1B and $p130^{Cas}$ are over-expressed in HeLa cells, EGF-induced RalA activation is increased in an additive manner. Therefore, $p130^{Cas}$ does not inhibit RalGPS1B, and in fact may act in concert with RalGPS1B under certain circumstances to activate Ral (e.g. in presence of growth factors or over-expressed EGFRs that are found in certain cancers).

Goi *et al.*, (2000) have determined that EGF and Ral activate c-Src, which subsequently activates downstream cortactin and STAT3. We show in quiescent A7r5 and HeLa cells that the basal and EGF-induced activation of c-Src and STAT3 is CaM-dependent, and in A7r5 cells, PLC-dependent, but in contrast to Ral activation, Ca^{2+}-independent. Although an increased concentration of free Ca^{2+} has been shown to be

sufficient for the activation of c-Src in non-myocyte cells (Rusanescu *et al.*, 1995), the above results indicate that this is not the case in EGF-stimulated A7r5 cells. Because c-Src and STAT3 activation requires PLC but not Ca^{2+}, these proteins may be activated by the other branch of the PLC/phosphoinositide pathway that involves the Ca^{2+}-independent, DAG-induced stimulation of novel PKC (e.g. PKCδ). Also, because c-Src and STAT3 activation requires CaM but not Ca^{2+}, these proteins must be able to be activated by very low levels of intracellular Ca^{2+}, or by Ca^{2+}-free CaM, apoCaM. This may involve the binding of CaM to the Ca^{2+}-independent binding domain of Ral (Clough *et al.*, 2002).

Both Ral- or EGF-activated c-Src can occur by a Ras-independent pathway, such as through Ca^{2+}, which allows c-Src to activate downstream targets without activating Ras and its effectors. The location of both Ral and c-Src in various cytoplasmic and membranous fractions of cells could explain part of these effects. Distinct receptor pathways could activate different pools of c-Src, whose access to different c-Src substrates differ. Selective Ral-dependent activation of c-Src substrates may allow c-Src to be activated without Ras/MAPK activation (Goi *et al.*, 2000). Like c-Src, Ral may be able to activate only a subset of potential downstream targets under distinct circumstances (Goi *et al.*, 2000) which may be determined by the specific intracellular location of Ral.

6.2.1 Model for EGF-induced Activation of Ral in A7r5 and HeLa Cells

We propose a model in A7r5 cells (Fig. 48) in which EGF binding to and activation of EGFR tyrosine kinase activity, recruits and activates adapter proteins and

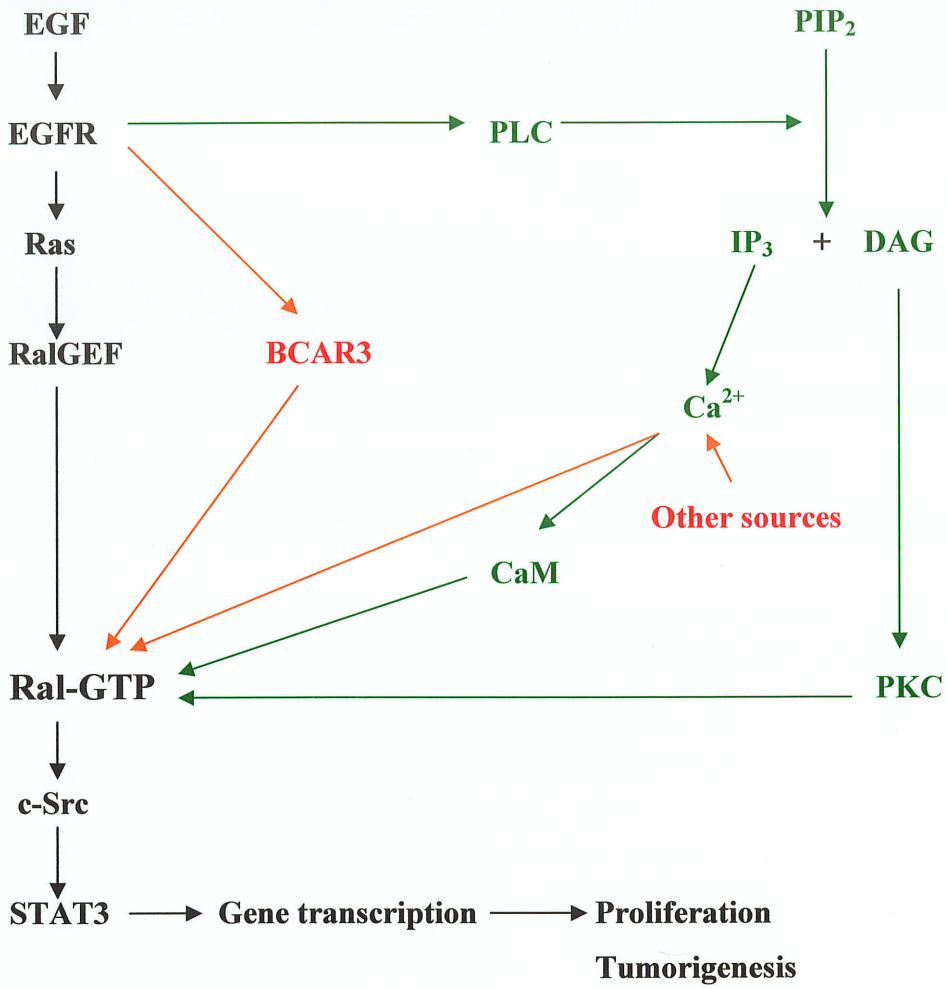

EGF
EGFR
Ras
RalGEF
BCAR3
PLC
PIP2
IP3 + DAG
Ca2+
Other sources
CaM
Ral-GTP
PKC
c-Src
STAT3
Gene transcription
Proliferation
Tumorigenesis

Figure 48

Fig. 48. Model depicting EGF-induced Ral activation in A7r5 and HeLa cells. In A7r5 cells, the EGF/EGFR complex induces the activation of both Ras and PLC pathways. Ras recruits RalGEF, which induces GTP binding to and activation of Ral. Activated EGFR also recruits and stimulates PLCγ to activate Ral via IP_3-induced increase in intracellular Ca^{2+} and via DAG-stimulated PKC. Apo-CaM or CaM at negligible intracellular Ca^{2+} concentrations also activates Ral. In HeLa cells, the EGF/EGFR complex primarily activates BCAR3/AND-34, which activates Ral in a Ca^{2+}-dependent manner. The PLCγ-mediated, IP_3-stimulated increase in Ca^{2+} is required for full RalB, but not RalA activation. In both HeLa and A7r5 cells, Ral-GTP activates c-Src, which in turn activates STAT3 and cortactin (Goi *et al.*, 2000). Green and red denote novel pathways in A7r5 and HeLa cells, respectively.

Ras in one branch of the Ral pathway, and PLCγ in a second branch. Ras-GTP brings Ras-binding RalGEFs to the plasma membrane where they activate RalA and RalB in a Ca^{2+}-, CaM- and PKCδ-dependent manner. The operation of both branches of this pathway need not be mutually exclusive. PLC cleaves PIP_2 to produce IP_3 and DAG. IP_3 induces a rise in intracellular Ca^{2+} from internal stores (Schlessinger, 2000; Prenzel *et al.*, 2001) which activates CaM, which in turn, binds to and activates Ral. CaM (or perhaps apo-CaM) can also activate Ral at very low or zero intracellular Ca^{2+} concentrations. In a third pathway, the DAG produced by PLCγ stimulates primarily the δ isoform of PKC, which also activates Ral. Both Ral proteins have a few consensus PKC phosphorylation sites, although, to date, none have been shown to be phosphorylated. EGF-activated Ral then induces the down-stream recruitment and activation of c-Src, and the subsequent activation of STAT3. This is the first record of CaM, PLC and PKC being involved in the EGF/Ral/STAT3 pathway. These results suggest that CaM plays an essential role in growth factor-mediated stimulation of STAT3-regulated gene transcription.

In HeLa cells our model (Fig. 48) predicts that EGF-stimulated EGFR recruits and activates adapter proteins and Ras at the plasma membrane in one branch of the Ral pathway, BCAR3/AND-34 in another branch, and PLCγ in a third branch. RalA and RalB are activated primarily by BCAR3/AND-34 in a Ca^{2+}-dependent manner. In a minor pathway, Ras-GTP may bring Ras-binding RalGEFs to the plasma membrane where they activate RalA and RalB in a Ca^{2+}-dependent manner. Although basal Ral activation is CaM-dependent, CaM-dependent pathways are only partially involved in EGF-induced RalA and RalB activation. In contrast to A7r5 cells, PKCδ is not involved in Ral activation by EGF. However, activation of RalB, but not RalA, requires PLC co-activated

by EGF. Therefore, to a greater extent than that which occurs with RalA, the maximal EGF-induced activation of RalB depends on the PLC-induced increase in intracellular Ca^{2+}, but not on activated PKC induced by the PLC-derived DAG. Because of the involvement of BCAR3/AND-34 in HeLa and MCF7 cells, we speculate that the use of Ras-independent RalGEFs by mitogens and growth factors in certain cell types may have implications in cell growth and tumorigenesis. It has been suggested (Goi *et al.*, 2000) that the upregulation and/or overactivity of a RalGEF may contribute to Ral-mediated tumorigenesis and metastasis. It is also apparent that $p130^{Cas}$ inhibits or stimulates Ral activation depending on cell type and environment. Therefore, we speculate that $p130^{Cas}$ can act as a tumour suppressor or an onco-protein, again depending on cell type and environment.

6.3 Ral Interacts with the β1 subunit of Na^+/K^+-ATPase and is Involved in the Signal Transducing Functions of the Na/K Pump

Ral is also involved in non-proliferative growth of cardiac myocytes, which can result in cardiac hypertrophy. Transfection of RalGDS and constitutively active RalGV23 mutant in cultured rat neonatal myocytes stimulated activity of proto-oncogenes and cardiac hypertrophic genes (Kawai *et al.*, 2003). As well, in cardiac hypertrophy, cardiotrophin-1 activity is increased, which results in activation of Ral in a STAT3-dependent manner (Kawai *et al.*, 2003). The partial inhibition of Na^+/K^+-ATPase in rat cardiac myocytes by ouabain up-regulates cardiac hypertrophic and proto-oncogenes that lead to hypertrophy. This occurs by protein-protein interactions at the cell membrane that stimulate various interacting signalling pathways, including Ras/MEK/ERK (Kometiani

et al, 1998, 2000; Tian *et* al, 2001; Haas *et al*, 2000, 2002). We speculated that Ral is involved in cardiac hypertrophy via the signal transducing pathways of ouabain-inhibited Na^+/K^+-ATPase. Using a yeast two-hybrid assay, we discovered that RalA and RalB bind directly to the $\beta 1$ subunit of Na^+/K^+-ATPase ($\beta 1$) *in vivo*. Subsequent *in vitro* binding and pull-down assays in human platelets and canine sarcolemmal membranes, as well as co-immunoprecipitation experiments in HeLa cells, verified this Ral/$\beta 1$ interaction. Therefore, we have shown for the first time in different cell types that $\beta 1$ binds directly to RalA and RalB *in vitro* and *in vivo*. The role of this interaction is unclear. Functionally, Ral may be involved in regulating the activity or expression of $\beta 1$, or Ral may be involved in trafficking and targeting of $\beta 1$ and the Na/K pump to the plasma membrane.

To study the role of Ral in the signal transducing pathways of Na^+/K^+-ATPase, we used A7r5 and HeLa cells. Linkage to signaling intermediates and pathways through protein-protein interactions is a common property of Na^+/K^+-ATPase in most, if not all cells. Ouabain-induced activation of the Ras/ERK pathway and generation of ROS has been demonstrated in A7r5 and HeLa cells (Haas *et al.*, 2000; Liu *et al.*, 2000). Importantly, effective ouabain concentrations in human HeLa cells are 2 to 3 orders of magnitude lower than those in rodent A7r5 cells. That this corresponds closely to the known sensitivities to ouabain of the α subunits in these cells (Haas *et al.*, 2000; Liu *et al.*, 2000; Mohammadi *et al.*, 2001; Tian *et al.*, 2001), establishes that ouabain's effects on signalling pathways do begin at the Na^+/K^+-ATPase, and are not due to unidentified ouabain interactions with other receptors (Xie and Askari, 2002). Also, HeLa and A7r5 cells contain little or no Na^+/Ca^{2+}-exchanger, and are therefore not subject to Ca^{2+} overload toxicity when treated with ouabain. Therefore, the demonstration of the

independence of the signal transducing role of Na^+/K^+-ATPase (e.g. from Ca^{2+} ions) using altered intracellular ion concentrations is easier in these cells than in cardiac myocytes (Liu *et al.*, 2000). We determined that short-term treatment with ouabain causes minimal but reproducible Ral activation over basal levels in quiescent A7r5 cells, but has no effect in heLa cells. This indicates that ouabain-induced inhibition of Na^+/K^+-ATPase minimally affects Ral activation. We also determined that the lack of Ral activation was not due to the lack of Na^+/Ca^{2+}-exchanger-induced rise in Ca^{2+} levels in these cells. Our results therefore show a difference between ouabain-induced Ral and Ras activation, because Ras is significantly activated by ouabain-induced inhibition of Na^+/K^+-ATPase (Kometiani *et al.*, 2000; Haas *et al.*, 2000, 2002).

Because Ca^{2+} is known to activate Ral (Hofer *et al.*, 1994, 1998; Urano *et al.*, 1996; Wolthuis *et al.*, 1998b; Zwartkruis *et al.*, 1998; M'Rabet *et al.*, 1999; Wang and Roufogalis, 1999; Park, 2001), yet our results show ouabain does not support increased activation of Ral in the presence of increased intracellular Ca^{2+}, it is possible that ouabain inhibits Ral activation by Ca^{2+}. This may be due to ouabain-stimulated pathways mopping up proteins (e.g. CaM) required for Ral activation. Alternatively, it may be that ouabain-stimulated pathways in the localized cellular environment do not contain the necessary substrates for Ral activation. Therefore, examining ouabain's effects on other pathways that activate Ral, we show that short-term (15 minutes) ouabain treatment decreased EGF-induced RalA and RalB activation in A7r5, but had no effect in HeLa cells. Because we have shown CaM is required for EGF-induced activation of Ral in A7r5 but not HeLa cells, we speculate that ouabain may inhibit the interaction between Ca^{2+}/CaM and Ral in cells. Therefore, this would inhibit Ral activation in A7r5 but not

HeLa cells. These results demonstrate that partial inhibition of Na^+/K^+-ATPase by ouabain treatment cross-reacts with other pathways in a cell-dependent manner. This may have implications in patients treated with cardiac glycosides.

Examining the effects of longer term ouabain treatment (to activate short-term, long-term and hypertrophic genes) on EGF-induced Ral activation, we show that pre-treatment of A7r5 and HeLa cells with ouabain for 24 hours caused almost total inhibition of basal RalA and RalB activation. We subsequently determined that this result was caused by markedly reduced RalA and RalB protein expression. This effect occurred in A7r5 cells even at 20% of the ouabain concentration commonly used in the literature. Our results suggest that in non-myocyte cells, inhibition of Na^+/K^+-ATPase by ouabain inhibits Ral expression, and conversely that a normal functioning pump maintains or up-regulates Ral expression.

It has been proposed that ouabain and other hypertrophic stimuli (Kometiani *et al.*, 2000) as well as various growth factors (Bhutada and Ismail-Beigi, 1991) cause increased $\beta 1$ expression. Therefore, $\beta 1$ is regulated by growth signals, and up-regulation of $\beta 1$ may represent a common feature of cell growth (Kometiani *et al.*, 2000). However, in contrast to Kometiani *et al.*, (2000), who found that up to 24 hours of ouabain treatment in rat neonatal cardiac myocytes caused an up-regulation of $\beta 1$ mRNA levels, we show that similar long-term ouabain treatment in A7r5 and HeLa cells caused marked reductions in the protein expression of $\beta 1$, as well as of Ras in A7r5 and HeLa cells, and of CaM in A7r5 cells. These contradictory results may be due to the different cell types used, such as due to the lack of ouabain-induced increase in intracellular Ca^{2+}. It is possible our results are due to the large swings in Na^+ and K^+ concentrations that occur in

ouabain-treated A7r5 and HeLa cells, but which cannot occur in cardiac myocytes without Ca^{2+}-induced cytotoxicity (Xie and Askari, 2002). Our results may also simply demonstrate that mRNA transcription need not correlate directly with protein translation since protein expression was not determined in the study of Kometiani *et al.* (2000). We also show that dominant-negative Ral has a sparing effect on ouabain-induced down-regulation of β1 expression in A7r5 but not HeLa cells. Therefore, in A7r5 cells, Ral enhances the negative effect of ouabain on β1 and Ras expression, and there are obviously feed-back loops regulating Ral, Ras and β1 expression. For example, long-term ouabain treatment down-regulates β1 and Ras protein expression, which is enhanced by Ral. However, Ral expression is decreased at the same time, which limits the negative effect of Ral on β1 and Ras protein expression, and thus describes a self-regulating feed-back loop. In cardiac myocytes, a down-regulation of Ras protein expression by ouabain would be a protective or feed-back mechanism to prevent prolonged activation of Ras and subsequent hypertrophic genes and cell growth. This would help protect patients on long-term cardiac glycoside treatment against developing cardiac hypertrophy. These results also suggest that endogenous ouabain and other cardiotonic steroids may affect Ras and Ral protein expression.

The effects of long-term ouabain are not due to effects on cell viability. We got similar results in A7r5 cells when the ouabain dose was reduced to 20 μM from the recommended 100 μM, which causes only about 30% inhibition of the Na/K pmp. The cells appeared morphologically normal, and it is known that cells which do not express the plasma membrane Na^+/Ca^{2+}-exchanger (e.g. HeLa, A7r5 cells) can tolerate complete inhibition of the transport function of Na^+/K^+-ATPase for hours, even though exhibiting

large changes in $[Na+]_i/[K+]_i$ ratio. This is because they are not subject to the toxic effects of Ca^{2+}-overload as would occur in cardiac myocytes (Xie and Askari, 2002).

In the absence of ouabain, constitutively active RalA causes an upregulation of $\beta 1$ expression after 24 hours in A7r5 cells. Therefore, overexpression of Ral increases $\beta 1$ expression in A7r5 cells. However, when A7r5 cells are treated with ouabain long-term, Ral has the opposite effect of decreasing $\beta 1$ expression. The difference would be due to other pathways being activated by ouabain. In HeLa cells, constitutively active RalA and RalB have no effect on $\beta 1$ expression. This is similar to the situation in HeLa cells treated with long-term ouabain, where Ral has no additive or inhibitory effect on ouabain-induced down-regulation of $\beta 1$ expression. These results show differential regulation of $\beta 1$ expression by Ral in A7r5 versus HeLa cells.

Ouabain's gene regulatory and hypertrophic effects are dependent on Ca^{2+}, CaM and PKC, which have been claimed to act upstream (Kometiani *et al.*, 2000) or downstream (Xie and Askari, 2002) of Ras. Ral is a CaM binding protein (Wang *et al.*, 1997; Clough *et al.*, 2002), and we have shown above that CaM is required for the thrombin-induced activation of Ral in human platelets (Clough *et al.*, 2002) and for the EGF-induced activation of Ral in A7r5 and MCF7 cells. Here we demonstrate in A7r5 and HeLa cells, that CaM is also required for activation of c-Src, the first molecule activated by ouabain-induced inhibition of Na^+/K^+-ATPase. This is the first record of CaM being involved in the ouabain-activated pathway upstream of c-Src. Because ouabain treatment activates c-Src (Haas *et al.*, 2000) in a CaM-dependent manner, and induces c-Src to form a complex with the α subunit of Na^+/K^+-ATPase (Haas *et al.*,

2002), we propose that ouabain's hypertrophic effects are mediated via c-Src through Ca^{2+} and CaM.

Ouabain treatment causes translocation of c-Src to the α subunit of Na^+/K^+-ATPase (Haas *et al.*, 2002) where it is activated (Haas *et al.*, 2000, 2002). Because we show Ral binds directly to the β1 subunit of Na^+/K^+-ATPase, and that CaM is involved in activation of c-Src by ouabain, we suspect that Ral is involved in the ouabain-induced, CaM-dependent activation of c-Src. Co-immunoprecipitation experiments demonstrate that RalB and c-Src do not form a complex in HeLa cells. However, this does not preclude their being weakly or transiently involved in a common complex (e.g. in ouabain-stimulated cells). We demonstrate in A7r5 and HeLa cells that dominant-negative Ral causes an increase in ouabain-induced c-Src activation. These results suggest that Ral inhibits ouabain-induced c-Src activation in non-myocytes. We propose that Ral, Na^+/K^+-ATPase, and c-Src form a functional complex in cells upon ouabain stimulation, with Ral acting as an inhibitory protein. In our proposed model, ouabain stimulates Na^+/K^+-ATPase and c-Src to form a complex. CaM is either part of the complex, or CaM-mediated pathways positively regulate the complex. As part of a negative feed-back loop, Ral binds β1 which dissociates CaM from the Na^+/K^+-ATPase/c-Src complex, or inhibits CaM-regulated pathways, thus inhibiting CaM-dependent c-Src activation by ouabain. This is the first report of Ral being directly involved in the ouabain-stimulated signal transducing pathway of Na^+/K^+-ATPase at the level of c-Src.

6.4 Conclusion

In conclusion, our results point to RalA and RalB being CaM binding proteins, and therefore being intimately involved in CaM-regulated pathways in different cell types, both proliferating and non-proliferating, and involving disparate signal transduction pathways. Because CaM and, in many cases its binding partner Ca^{2+}, regulate many important cellular processes, Ral-GTPases are now functionally implicated in a wide range of signalling pathways. However, it is important to note that the regulation of Ral by CaM depends on cell type. This suggests that there are factors in cells that inhibit the Ral/CaM interaction, thus regulating Ral activation. It is also possible that access to substrates upon stimulation of certain receptors is limited in some cell types.

Our research specifically demonstrates the central role of c-Src in signalling pathways, and that Ral and CaM may be required in many instances for c-Src regulation. A strong correlation between Ral, CaM and c-Src activity has now been shown in proliferative and non-proliferative cell growth, involving cancer and non-cancer cells, as well as cardiac myocytes. Therefore, Ral, CaM and c-Src are pivotal molecules in regulating diverse biochemical pathways involved in cell growth, proliferation, oncogenesis and cardiac hypertrophy.

6.5 Future Directions

Further study is needed to confirm and advance all the conclusions of our results. It is important to determine the exact amino acid residues in Ral that make up the CaM BDs. This could be achieved by *in vitro* mutagenesis techniques, followed by binding

assays as described here. These studies would also accurately verify the presence of N-terminal Ca^{2+}-independent and C-terminal Ca^{2+}-dependent CaM BDs in RalA and RalB. The suspected differential binding affinity between CaM and RalA and RalB could be verified quantitatively by affinity binding studies, such as by use of surface plasmon resonance assay. *In vitro* mutagenesis techniques and CaM binding assays could also be used to examine the C-terminal CaM binding inhibitory region in RalB. To check for Ca^{2+}-induced conformational changes in this region would require 3D structure analysis. The identity of CaM binding proteins and CaM-regulated pathways that are involved with Ral remain to be determined. Known CaMBPs could be examined using co-immunoprecipitation and *in vitro* and *in vivo* binding assays described here. Once CaM pathways are known, chemical inhibitors supported by dominant-negative and constitutively active transfection experiments, plus small interfering RNA assays, could be employed to examine the effect of CaM on downstream Ral effector pathways and visa versa.

To further determine the role of Ral in cell proliferation and oncogenic transformation, it will be necessary to determine how other growth factors and mitogens affect the activation of Ral in a variety of cells, including cancer- and non-cancer-derived cells, as well as non-immortalized cells. Such experiments could include transfection experiments with Ral and RalGEF mutants to study cell proliferation and anchorage-independent growth. Genetic techniques to look for Ral and RalGEF mutations or polymorphisms in cancer cells may be warranted. In this way, a protein or mutation may be discovered that is specific to cancer and cancer-derived cells, or to a specific type of cancer. This would then be a target for cancer therapy. Similar experiments should be

performed with $p130^{Cas}$ to study its possible tumour-suppressor and onco-protein properties, as well as its possibility as a target of therapeutic agents in cancer treatment. Such experiments are needed to see if results in tissue cell cultures can be applied to cancer and non-cancer cells *in situ*.

Identification of additional Ral-interacting proteins is required to expand the known physiological functions of Ral. The screening of the human testis cDNA expression library with RalB resulted in several positive colonies. Therefore, research on these additional candidate proteins should help define new functions for Ral.

Further study is required on the involvement of Ral in the ouabain-induced signal transduction pathways mediated through c-Src, such as in cardiac myocytes. This would help determine the molecular mechanisms involved in ouabain-induced cardiac hypertrophy, and the cellular effects of endogenous cardiotonic steroids.

Several lines of evidence suggest that the α-β Na^+/K^+-ATPase complex is functional soon after it is formed in the ER (Tamkun and Fambrough, 1986; Caplan *et al.*, 1990; Geering *et al.*, 1987; Chow and Forte, 1995). After assembly in the ER, the α-β heterodimers move through the Golgi network, where further post-assembly processing (sugar modification) takes place and distinct paths are set up for trafficking of the Na^+/K^+-ATPase to the basolateral plasma membrane via transport vesicles (Chow and Forte, 1995). It would also be of considerable interest to determine whether and how Ral is involved in trafficking and targeting of the Na/K pump to the plasma membrane, and what role this plays in cardiac hypertrophy and physiological function of the pump. It will also be important to determine if Na^+/K^+-ATPase has important physiological functions within the biosynthetic-secretory pathway before it reaches the plasma membrane. For

example, Na^+/K^+-ATPase may be stimulated to interact with c-Src within the biosynthetic-secretory pathway. Both c-Src and Ral co-localize to intracellular vesicles (Bjorge *et al.*, 2000; Goi *et al.*, 2000), and our results suggest both are involved in the signal transducing function of Na^+/K^+-ATPase. Ral may interact with both β1 directly and c-Src indirectly at intracellular vesicles. The cytoplasmic portion of the Src-activated EGFR would then be able to serve as a scaffold for the recruitment of adaptor proteins and Ras, and the activation of the Ras/ERK cascade (Schlaepfer *et al.*, 1998; Eguchi *et al.*, 1998; Tice *et al.*, 1999). Therefore, it should be determined whether Na^+/K^+-ATPase is also linked to the Ras/ERK cascade (Haas *et al.*, 2000) through vesicular c-Src and EGFR. It will be important to specifically determine Na^+/K^+-ATPase activity within intracellular vesicles of cardiac myocytes and non-myocyte cells, and to determine the effects on such activity by glycosides, Ral and changes in intracellular ion (Na^+, K^+, Ca^{2+}) concentrations. Our results also suggest there should be studies into whether partial inhibition of Na^+/K^+-ATPase by cardiac glycosides inhibits other pathways that activate Ral.

Research on the long-term effects of cardiac glycoside treatment on signal transduction pathways and on the state of the Na/K pump is warranted. An emerging topic that needs further study is the function of endogenous cardiotonic steroids in myocyte and non-myocyte cells. Because ouabain and related compounds are endogenous steroid hormones (Schoner, 2002), it is also important to study the effects of physiological concentrations of these cardiotonic agents. What effects long-term glycoside treatment has on endogenous glycoside expression levels and function should also be determined.

Answers to the points raised here will increase our knowledge on the role of Ral-GTPases in health and disease.

7. SUMMARY

1. *In vitro* binding assays with [^{35}S]Met-labelled proteins and GST-fusion proteins show recombinant RalA and RalB and CaM interact specifically *in vitro* in a Ca^{2+}-dependent manner.
2. Endogenous CaM and Ral co-immunoprecipitate in human platelets.
3. Y2H assays show that CaM interacts specifically with both RalA and RalB *in vivo* in a eukaryotic system. RalA appears to bind CaM more readily than RalB.
4. *In vitro* binding assays with [^{35}S]Met-labelled proteins and GST- and Sepharose-fusion proteins demonstrate that full-length and C-terminally truncated RalA and RalB bind CaM in the presence of Ca^{2+} *in vitro*. C-terminally truncated RalB binds CaM more readily than full-length RalB, and in the absence of Ca^{2+}. This suggests that the C-terminal region of RalB is inhibitory to CaM binding, and that RalB has another CaM BD that is Ca^{2+}-independent.
5. *In vitro* binding assays with [^{35}S]Met-labelled proteins show that RalA and RalB have an N-terminal Ca^{2+}-independent and a C-terminal Ca^{2+}-dependent CaM BD.
6. GST-RRBD pull-down experiments determine that CaM is required for the thrombin-induced activation of RalA and RalB in human platelets.
7. GST-RRBD pull-down experiments with chemical inhibitors determine that (a) CaM is required for the EGF-induced activation of Ral in A7r5 and MCF7 cells but not in HeLa cells; (b) Ca^{2+} is required for the basal and EGF-induced activation of Ral in A7r5 and HeLa cells; (c) PLC is required for the basal and EGF-induced activation of Ral in A7r5 cells, and of RalB, but not RalA, in MCF7 and HeLa cells; (d) PKCδ is involved in the EGF-induced activation of Ral in

A7r5 cells, but not in HeLa cells; (e) PMA induces activation of Ral in A7r5 and HeLa cells via PKCδ in a Ca^{2+}/CaM-dependent manner.

8. Dominant-negative Ral transfection experiments show that Ras-dependent Ral-GEFs are required for basal and EGF-induced activation of RalA in A7r5 but not HeLa cells.

9. Dominant-negative Ral and $p130^{Cas}$ transfection experiments show that the Ras-independent RalGEF, BCAR3/AND-34, is required for the EGF-induced activation of RalA in HeLa but not A7r5 cells, and that BCAR1/$p130^{Cas}$ is stimulatory to Ral activation in MCF7 cells. Therefore, the RalGEFs used in signal transduction depends on cell type and environment.

10. Western blotting with phosphor-specific antibodies determined that EGF-induced activation of c-Src and STAT3 requires CaM but not Ca^{2+} in A7r5 and HeLa cells.

11. Screening of a human testis cDNA expression library and Y2H assays show that β1 subunit of Na^+/K^+-ATPase interacts with RalA and RalB *in vivo* in a eukaryotic system.

12. GST- and Sepharose fusion-protein pull-down assays determine that endogenous β1 interacts with recombinant Ral, and visa versa, in (a) human platelets and (b) CSM preparations.

13. *In vitro* binding assays show that recombinant *in vitro* transcribed/translated [^{35}S]Met-labelled β1 interacts with GST-RalA and GST-RalB *in vitro.*

14. Ral immunoprecipitates with β1 but not c-Src in HeLa cells.

15. GST-RRBD pull-down assays of activated Ral show that ouabain-induced inhibition of Na^+/K^+-ATPase has a minimal effect on the basal activation state of

Ral, and that increased intracellular Ca^{2+} does not enhance Ral activation in the presence of ouabain. Suggests ouabain inhibits Ca^{2+}–induced activation of Ral.

16. Inhibition of Na^+/K^+-ATPase activity by short-term ouabain treatment inhibits EGF-induced Ral activation in A7r5 but not HeLa cells, while long-term treatment inhibits RalA and RalB activation by EGF in A7r5 and HeLa cells.

17. Inhibition of Na^+/K^+-ATPase activity by long-term ouabain treatment inhibits (a) Ral, (b) Ras, (c) CaM and (d) β1 protein expression, an effect that is enhanced in A7r5 but not HeLa cells by Ral.

18. Western blotting determined that constitutively active Ral upregulates β1 protein expression in A7r5 but not HeLa cells.

19. Western blotting with phosphor-specific c-Src antibody shows that CaM is required for the ouabain-induced activation of c-Src in HeLa and A7r5 cells, an effect that is inhibited by Ral. First report of CaM acting upstream of c-Src, and of Ral being involved in the signal transduction pathway of the ouabain-inhibited Na/K pump.

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