ESTABLISHMENT AND CHARACTERIZATION OF MCF-7 HUMAN BREAST CANCER CELL CLONES EXPRESSING AN INTRACELLULAR FORM OF INSULIN-LIKE GROWTH FACTOR II (IGF-II)

by
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"Assuredly the creation of the heavens and the earth is a greater (matter) than the creation of men: yet most men understand not."

(Al-Quran 40:57)

ABSTRACT

Insulin-like growth factor II (IGF-II) has been proposed to be a critical estrogen-induced mitogen which regulates breast cancer cell growth. The constitutive expression of IGF-II may play an important role in the mechanism underlying the progression of tumor cells to hormone independent growth. In addition to autocrine and paracrine influences on breast cancer, the abundance of intracellular receptors for IGF-II (IGF-II/M6P receptors) has led to the suggestion that IGF-II may act intracellularily. In order to test whether IGF-II is able to act via an intracrine mechanism, MCF-7 cells were transfected with an expression vector containing the coding sequence for a mutant preIGF-II (no E-domain) peptide with a carboxyl-terminus KDEL-endoplasmic reticulum retention signal. MCF-7 cells do not express endogenous IGF-I or IGF-II which would interfere with the testing of our hypothesis. These cells do, however, express both IGF type 1 and type 2 receptors and proliferated in serum-free medium when exposed to physiological concentrations of IGF-II. IGF-II responsiveness and the lack of endogenous IGFs are necessary properties of an appropriate host cell to test our hypothesis. Following transfection and selection in G418 containing medium, seven of the twelve isolated single cell clones expressed the mutant IGF-II transcript. Radioimmunoassay on cellular extracts showed that IGF-II-KDEL was accumulated abundantly intracellularly, with values ranging from 30 to 800 ng/mg protein. IGF-II-KDEL was not detected in the media, indicating that the retention signal (KDEL) functioned effectively. The expression of IGF-II-KDEL in MCF-7 cells, however, resulted in cell growth characteristics comparable to wild-type MCF-7 cells or control clones transfected with the same expression vector without insert. The addition of exogenous IGF-II resulted in significant growth of our clones. Thus, IGF-II-KDEL was unable to act intracellularly. While assaying our IGF-II-KDEL clones for growth, we observed that one of our clones (clone 12) was able to grow without any serum or exogenous growth factor, although this clone produced only intermediate levels (30-140 ng/mg) of IGF-II-KDEL. This growth could not be attributed to intracellular IGF-II-KDEL since several other IGF-II-KDEL expressing clones displayed growth comparable to control cells. Further analysis of this clone is necessary to gain insights into the early mechanisms involved in progression from hormone dependence to hormone independence.

LIST OF ABBREVIATIONS

ALS	acid-labile subunit
AMP/cAMP	adenosine monophosphate/cyclic AMP
ATP/dATP	adenosine triphosphate/deoxyATP
BiP	immunoglobulin heavy chain-binding protein
bp	base pairs
BSA	bovine serum albumin
°C	degrees centigrade
Ca/CaCl	calcium/calcium chloride
CD	cation-dependent
cDNA	complementary DNA
CI	cation-independent
CMV	cytomegalovirus
CO_2	carbon dioxide
CRE/CREB	cAMP response element/CRE binding protein
CTP/dCTP	cytosine triphosphate/deoxyCTP
DAG	1,2-diacylglycerol
DES	diethylstilbestrol
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	estrogen receptor/endoplasmic reticulum
EtBr	ethidium bromide
FBS/csFBS	fetal bovine serum/charcoal stripped FBS

FGF	fibroblast growth factor
g/mg/μg/ng	grams/millig/microg/nanog
GAP	GTP activating protein
GH	growth hormone
GRB	gel running buffer
GTP/dGTP	guanosine triphosphate/deoxyGTP
H	hydrogen
hr	hour
IgA	immunoglobulin A
IGF/IGFBP	insulin-like growth factor/IGF binding protein
IL	interleukin
IP_3	inositol 1,4,5-triphosphate
IRS	insulin receptor substrate
kDa	kilo Dalton
KAc	potassium acetate
l/ml/μl	liters/millil/microl
LDL	low density lipoproteins
M6P/Man-6-P	mannose-6-phosphate
$MgCl_2$	magnesium chloride
MPR	M6P receptor
Mr	molecular weight
MSA	multiplication-stimulating activity
MT	metallothionein
Na/NaCl/NaOH/NaAc	sodium/-chloride/-hydroxide/-acetate
NCI	National Cancer Institute
NEO	neomycin
NGF	nerve growth factor
NSILA	non-suppressible insulin-like activity
ORF	open reading-frame
PBS	phosphate-buffered saline

PCR/RT-PCR	polymerase chain reaction/reverse transcription-
PDGF	platelet-derived growth factor
PDI	protein disulphide isomerase
PI/PIP_2	phosphatidyl inositol/PI 4,5-biphosphate
PKA	protein kinase A
PLC	phospholipase C
RIA	radioimmunnoassay
RNA/mRNA	ribonucleic acid/messenger RNA
RPM	revolutions per minute
TBE	Tris borate EDTA buffer
$t_{1/2}$	half-life
TE	tris-EDTA
TGF-α/TGF-β	transforming growth factor-alpha/-beta
TTP/dTTP	thymidine triphosphate/deoxyTTP
$ZnCl_2$	zinc chloride
%	percent

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INTRODUCTION

A. The Breast Epithelium

Normal Breast Development and Function: Endocrine Regulation

The breast originates from the embryonic surface epithelium as do all other derivatives of the skin, such as hair, subaceous glands, and sweat glands. However, in contrast to most exocrine glands which develop an entire secretory system long before birth, the mammary gland develops only a duct system and remains in this incomplete state until puberty. The majority of breast development occurs during four distinct periods of dramatic differentiation resulting in extensive structural change, separated by "resting periods" of little change.

Embryology and the Immature Breast

i) The fifth week of human fetal development is characterized by the appearance of the ectodermal primitive milk streak of thickened epithelium on each side of the ventral midline, extending from the axilla to the groin. This represents the first signs of mammary development (Vorherr, 1974). By 7 weeks a mammary ridge forms in the thoracic area while the rest of the primitive milk streak regresses. This is followed by thickening and invagination of the mammary ridge into the underlying chest wall mesenchyme. In addition, peripheral nerves, blood vessels and lymphatic channels begin to form within the mesenchymal tissue. The embryonic period ends at 8 weeks, and the more human appearence of the embryo, with distinct eyes, face, and limbs, appears at the beginning of the following fetal period (O'Rahilly, 1983; Moore, 1988).

ii) No further differentiation occurs until the fourth month, but until then the mammary primordium enlarges slowly. By 12 to 14 weeks, mesenchymal cells differentiate to form

the smooth muscle fibers of the nipple and areola. At 16 weeks, epithelial buds develop, then branch to form 15 to 25 bands of epithelium branching out into the subcutaneous tissue and form the anlage of the ductal system and the secretory alveoli (Hughes, 1950). The secondary mammary anlage then develops with complete formation of sweat glands and only partial differentiation of hair follicles and subcutaneous glands.

Literature detailing how prenatal hormonal environment affects mammogenesis (the growth and differentiation of the mammary gland) in rodents is vast (Topper and Freeman, 1980 and references within). Treatment of male mice with antiandrogens or inhibitors of 5-α reductase (the enzyme that converts testosterone to DHT) results in the development of female mammary glands (Imperto-McGinley, 1986). Thus, fetal gonadal secretions determine the sexually dimorphic pattern of mammary growth in rodents.

There are very few studies using human breast tissue. Consequently, hormonal influences on early breast growth and differentiation are less well understood in humans. Breast development to this stage, however, is thought to be hormone independent. Placental sex hormones entering the fetal circulation during the third trimester of pregnancy are required to induce canalization of branched epithelial tissue (Ceriani, 1974), which differentiate into lobular and acinar structures at 32 to 40 weeks and regress after birth. There is increase growth of fat and vascularized connective tissue beginning at 20 weeks, and consequently, mammary gland mass increases four fold at 32 to 40 weeks. At this point the nipple-areolar complex also develops and becomes pigmented. During the neonatal period and early childhood, there is only slight longitudinal growth and branching of the primary ducts. As a result of maternal sex hormones that crossed the placenta late in pregnancy, the baby's breasts are usually swollen and a discharge of colostral milk (or "witch's milk") by the immature mammary ducts of the newborn of both sexes may be present a few days after birth. As these hormones diminish in the baby's circulation, the breast tissue regresses.

iii) The ovarian hormones, estrogen and progesterone, activated and regulated by follicle stimulating and luteinizing hormones from the pituitary gland, cause the female breast to rapidly enlarge. This is in part due to branching and extension of the mammary duct system and increase in the volume of connective tissue necessary to provide support for developing ducts; however, it is mostly due to adipose deposition. Elevation and pigmentation of the female areola mark the start of sexual differentiation of the breast. Animal experiments show that the growth of ductal epithelial and myoepithelial cells are influenced particularly by estrogen, while estrogen and progesterone together stimulate ductular-lobular-alveolar development of mammary tissue (Ceriani, 1974; Porter, 1974; Monaghan, 1990).

The mature female breast is comprised of skin, subcutaneous tissue, and breast tissue which is made up of both parachymal and stromal elements. The parachyma is composed of extensively branched ducts surrounded by collagen fibers, similar to a tree-like structure, converging at the nipple. The ductal system consists of the relatively large lactiferous sinus (a collecting duct leading to the ampulla opening at the breast surface) which continues to the main conducting lactiferous duct (or interlobular ducts), and ends with the finely branching terminal intralobular ducts. Each duct drains a lobe made up of 20 to 40 lobules, further composed of grape-like clusters of 10 to 100 alveoli (Parks, 1959). Ducts are surrounded by connective tissue and myoepithelial cells which respond to oxytocin. Adipose and connective tissue, separating and supporting numerous glandular elements, along with blood vessels, nerves and lymphatics, make up the components of stroma and subcutaneous tissue of the breast. The skin of the breast is thin and contains hair follicles, sweat glands, subaceous glands, and large accessory areolar glands of Montgomery, which lubricate the nipple for nursing and are also capable of secreting milk. These glands represent an intermediate stage between sweat and mammary glands on the surface of the areola. The nipple and surrounding pigmented areola are made of keratinized squamous epithelial cells. At the undersurface lies both longitudinal and

circular muscle fibers responsible for the erection of the nipple in response to stimulation. The nipple also contains numerous sensory nerve innervations whereby a suckling stimulation would trigger the release of milk and maintain glandular differentiation which is essential for continued lactation.

The breast has a liberal supply of blood vessels, nerves, and lymphatic vessels (Cunningham 1977). The arteries and their branches deliver the necessary nutrition and hormones for breast function and physiological changes that are closely associated with the endocrine system of the body. Many veins are important for the removal of waste products. The lymphatic system is very important. Breast tissue is constantly bathed with lymph fluid, which removes waste products of metabolism and milk production, along with dead cells via the lymph vessels. These unidirectional vessels empty mostly into auxillary lymph nodes present in the armpit, but also to other lymph nodes situated under the breast bone, above the collarbone, and along the side of the chest wall.

After menarche, ovarian estrogen during the menstrual cycle is responsible for moderate ductal growth, whereas cyclic levels of progesterone cause proliferation and regression of secretory acini budding from the terminals of intralobular ducts (Fanger and Ree, 1974; Longacre and Bartow, 1986). Estrogen is essential for mammary gland development (Nandi, 1958) but has been hypothesized to act indirectly through systematically acting substances such as growth factors. Studies with mouse and human normal mammary epithelial cells *in vitro* support this hypothesis (Yang et al, 1980; Stampfer and Bartley, 1987). Low-density cultures require multiple hormones (Hammond et al, 1984; Stampfer and Bartley, 1987) and estrogen induced proliferation of epithelial cells has been observed only when cocultured with mammary stromal cells (McGarth, 1983; Haslam and Levely, 1985). These studies suggest a close interaction between stromal and epithelial components. The significance of local growth factors on the growth regulation of these cells is becoming more apparent.

Normal human mammary epithelial cells rapidly proliferating in culture express high

levels of TGF-α and its receptor (Stampfer and Bartley, 1986; LeVay-Young, 1987; Rudland, 1987). TGF-α and EGF are able to promote local lobulo-alveolar development *in vitro* (Vonderhaar, 1987, 1988) and *in vivo* (Tonelli and Sorof, 1980; Vonderhaar, 1984). FGF is involved in maintaining mouse and human mammary myoepithelial and epithelial cells (Hammond et al, 1984; Smith et al, 1984). Unlike most growth factors, TGF-β is growth inhibitory and causes the differentiation of normal breast epithelial cultures (Walker-Jones et al, 1988; Walker-Jones et al, 1990) along with most other epithelial cells (Tucker et al, 1984; Roberts et al, 1985; Chakrapbarty et al, 1988). Slow-release of TGF-β near developing mammary ducts results in complete cessation of mammary ductal development in neonatal mice without any visible effects on surrounding stromal tissue or on more distant mammary glands (Silberstein and Daniel, 1987). TGF-β may act with other growth factors to delicately balance the process of human mammary development.

Although the female breast is mature in terms of its external contours, the final development of the duct system and secretory activity begins during the first trimester of pregnancy (Drife, 1986).

Pregnancy and Postpartum Period

iv) In addition to elevated levels of estrogen and progesterone during pregnancy (Tulchinsky et al, 1972), final breast maturation is dependent on the presence of prolactin; the placental lactogenic hormone, somatomammotropin; adrenal corticoids (cortisol); and insulin, in preparation for lactation (Salazar and Robon, 1974; Lyons et al, 1958; Cowie et al, 1980). The process of lactation occurs in three phases: (i) mammogenesis, the growth of the mammary gland; (ii) lactogenesis, the initiation of milk production; and (iii) galactopoiesis, the maintenance of established lactation. The levels of prolactin (Tyson et al, 1972; Kletzky et al, 1985) and a homologous placental product, placental lactogen (Spellacy and Buhi, 1969; Braunstein et al, 1980), in the blood begin increasing steadily at 8 weeks and peak prior to parturition. Both hormones induce mammogenesis

(Turkington, 1972), with prolactin being the more potent of the two peptides. Although growth hormone is lactogenic in some animals (Cowie et al, 1980), growth hormone is not necessary for mammogenesis or lactogenesis in humans since normal mammogenesis and lactation occur in women with growth hormone deficiency (Tyson et al, 1972; Rimoin et al, 1968).

Under the influence of corpus luteal estrogen and progesterone in the first trimester of pregnancy, rapid proliferation and differentiation of intralobular ductal epithelium begins to replace adipose and connective tissue with the development of lobules (Voherr, 1974; Beck et al, 1985). During the second trimester, estrogen and progesterone from the placenta, along with prolactin, continue to induce epithelial cells of the lobules to complete their differentiation in order to form active exocrine glands. At this point swollen ductal ends hollow out and become round alveoli (acini) as they begin to fill with colloid secreted by the newly formed epithelial lining cells. Initial production of colostrum is induced by placental lactogen (Lawrence, 1989). Differentiation of the terminal ducts into acini have been mimicked in culture systems by the addition of exogenous estrogen and progesterone (Robyn et al, 1986). During the last trimester, the secretory cells fill with oil droplets, and the alveoli are distended with a proteinaceous secretion termed colostrum. Myoepithelial cell hypertrophy and alveolar colostrum accumulation account for increased breast size (Ferguson and Anderson, 1983).

The lactogenic effects of prolactin, mediated through receptors on the mammary secretory cell surface of the alveoli, induce extraction of water, lactose, amino acids, fats, vitamins, minerals, and other components from the mother's bloodstream, and convert them to milk (Riordan, 1985). Prolactin levels increase steadily throughout gestation, and enzymes specific for milk synthesis and milk proteins appear in breast secretions before parturition (Rosen et al, 1986). However, because of the inhibitory effects of estrogen on the number of prolactin receptors, and progesterone on the biosynthesis of milk components, lactogenesis does not occur (Topper and Freeman, 1980).

After parturition, the decline of circulating estrogen and progesterone allows the full lactogenic effects of prolactin. The initial secretion, colostrum, is a thin yellowish fluid composed of lactoglobulin, which is identical to blood immunoglobulins, as well as fatty acids, phospholipids, vitamins, and lactalbumin which has considerable nutritional value. Colostrum may be an important factor in producing early immunocompetence in the newborn, since the lactoglobulin content (predominantly immunoglobulin IgA) is complemented by maternal lymphocytes, macrophages, and necessary proteins (Salazar and Robon, 1974; Committee on Nutrition, 1981; Karra et al, 1988).

Three or four days postpartum, true milk, comprised of a suspension of casein, α-lactalbumin, and β-lactoglobulin proteins along with fat in a lactose-mineral solution, is produced under the stimulation of pituitary prolactin (Lyons, 1942). A plethora of growth factors also make-up a component of human milk (Noda et al, 1984). Emulsified fats and calcium caseinate give milk its white color, whereas the yellow colored emulsion is due to the presence of carotenoids. Prolactin stimulated milk production requires the presence of insulin, growth hormone, thyroxine and the corticosteroid hormone derivatives, cortisol or hydrocortisone, which supplement prolactin's effect. TGF-β_1 and TGF-β_2 induce the synthesis of milk fat globule antigen (Walker-Jones et al, 1988). The corticosteroids increase the half life of milk protein mRNA (Rosen et al, 1986).

The maintenance of established lactation and milk removal by periodic suckling are closely associated processes. Suckling by an infant stimulates sensory nerve endings in the nipple-areolar complex resulting in release of oxytocin from the posterior pituitary and prolactin from the anterior pituitary (McNeilly et al, 1983; Johnston and Amico, 1986). In the rat, suckling also begins stimulating the replacement of secreted prolactin by inducing transcription of prolactin mRNA in the pituitary (Lee et al, 1989). Oxytocin released into the circulation then causes contraction of the smooth muscle components of the effector myoepithelial cells, resulting in the ejection of milk from the alveoli into the lactiferous ducts and sinus (Tucker, 1979; Grosvenor and Mena, 1974), thereby completing the *milk ejection reflex* or *let-down reflex.*

Once breast-feeding has stopped and milk is no longer removed, the cessation of lactation due to lack of prolactin and oxytocin leads to regressive changes. Similar results are observed when prolactin secretion is inhibited pharmacologically (Weinstein et al, 1976; Venturini et al, 1981). Connective tissue begins to replace most glandular tissue elements as secretory acini regress and degenerate. Correspondingly, interlobular connective tissue again becomes dominant as the breast returns to its essentially inactive status (Cowie et al, 1980; Salazar and Robon, 1974).

Senescence

Further regression of glandular and ductular epithelial structure, resulting in profound changes of the breast, parallels the decline of ovarian secretions of estrogen and progesterone at menopause. The breast usually becomes larger because of an increase in the amount of fat, and the involution of glandular substance takes place. The duct system remains but lobules disappear. Adipose and stromal tissue replace the parenchyma of the breast.

As women reach middle age, they often notice that their breasts seem lumpier or more nodular than when they were younger. This lumpiness is most prominent during the premenstrual phase of the monthly cycle and regresses with menstruation as estrogen levels diminish. Before the proliferative breast tissue can be completely reabsorbed, rising estrogen levels once again stimulate growth. Repetition of growth and regression in this manner during the menstrual cycle, without interuption by pregnancy and breast feeding, results in increased lumpiness experienced by many women in their thirties and early forties. Lumps remaining after menstruation may either be benign or malignant and should be investigated (Townsend, 1980). In terms of diseases of the breast, the cyclic capacity for growth and differentiation of the terminal ducts during normal menstrual cycling also presents a potential for the triggering of abnormal mitotic hyperactivity, including carcinomatous proliferation of duct epithelium. In this sense, the terminal intralobular ducts and normally unfinished alveolar ends of the duct system present a site

of unusual sensitivity and tissue vulnerability.

Human Breast Cancer

A General Introduction

In order to facilitate our understanding of breast cancer, we need to know the factors controlling normal cell growth; but first, we need to know exactly what cancer is. Cancer is often seen as a single entity, when in reality it is not one disease; rather it is more than a hundred different diseases. Similarly, there are many different types of breast cancer. What all cancers have in common is abnormal and uncontrolled cell growth which can invade and destroy normal body tissue.

Ordinarily, the division of the various kinds of cells in the body is orderly and controlled, and can be generally categorized into three basic growth patterns. The first category of cells have well-defined life cycles, such as stem cells, which continuously reproduce and replace themselves as long as we are alive. Stem cells turn over rapidly to return blood hematocrit to normal, relatively soon after we donate blood, and are responsible for constant replacement of blood cells dying by apoptosis. The second family of cells reproduce a limited number of times, such as cells found in organs. Once the kidney or heart has reached a "mature" size there is no need for further growth. Finally, the third type of cell, like nerve cells, is so specialized that it does not regenerate at all; which is why nerve damage is usually permanent.

Cancer cells are no longer bound by growth restraints, and as a result, continue to proliferate uncontrollably. Distinct characteristics, such as predominating cell type, site of origin, rate or pattern of growth, and extent of spread, are the main features used to distinguish one type of cancer from another. This is particularly true for breast cancer. Breast cancers may grow very slowly, be confined to a localized area of the breast, and cause no pain or symptoms until they are far advanced. Other localized tumors grow

much more rapidly, while still others spread to distant areas of the body. Such uncontrolled cell division can develop into a lump or tumor, which can be either benign or malignant.

Benign refers to a lump or tumor whose cells do not invade neighbouring tissue. However, if left untreated and allowed to continue to grow, it may put pressure on surrounding organs and interfere with normal organ and body functions. Benign tumors rarely lead to the death of a patient. The tissue of tumor origin can be connective tissue, such as fat or fibrous material (lipoma), or glandular tissue (adenoma). Benign tumors are described by adding the suffix -oma to the name of the tissue where it originates.

A malignant lump or tumor contains cells that have the ability to spread beyond their site of origin, and if left untreated would invade and destroy surrounding tissue. Cancers of connective tissue are very rare and are indicated by the suffix sarcoma, whereas malignant tumors of epithelial (or glandular) tissue are indicated by the suffix carcinoma. Most breast cancers arise from the epithelial cells lining the milk ducts or the milk producing lobules at the duct terminals (Azzopardi, 1979; McDivitt et al, 1968; World Health Organization, 1982). Epithelial cells have a high rate of turnover, and are constantly being shed and replaced with new cells. Malignant tumors of epithelial origin are termed adenocarcinoma. Other sites in the breast where cancer may develop include the fat cells (liposarcoma), lymph tissue (lymphoma), connective tissue (fibrosarcoma), or muscle fibers (rhabdosarcomas), but these are rare.

Malignant tumors are of two types: non-invasive and invasive. Malignant cell growth confined to a small area without spreading to adjacent tissue is referred to as non-invasive or "carcinoma *in situ*". Approximately six percent of all breast cancers are non-invasive, and are evenly divided between ductal carcinoma *in situ* and lobular carcinoma *in situ*. There is a high probability that a carcinoma *in situ* will eventually evolve into an invasive cancer; however, if the lump is excised in its early stages of evolution, the outlook for the patient is excellent. About ninety-four percent of all breast cancers are invasive. That

is, malignant cells that have broken through their site of origin (such as a breast duct or a lobule), have spread to surrounding tissue, and are capable of metastasizing to another part of the body. Invasive lobular cancer is relatively unusual, accounting for about eight percent of malignant breast tumors. Unfortunately, invasive lobular cancers have often spread to the lymph nodes by the time of diagnosis, worsening the overall prognosis. The most frequently encountered type of breast cancer, invasive ductal carcinoma, accounts for ninety percent of all malignant tumors and will be implied when generally referring to breast cancer from now on. Similarly, in invasive lobular cancer, spreading to axillary lymph nodes is common and again results in a poor prognosis.

All cancers discussed thus far are primary tumors. Primary cancers develop from the original cell transformation in an organ, such as breast or lung, and is not an extension of, or spreading from, a cancer at another site. The great danger of cancer is that secondary cancers may develop. Cells eventually break away from the original primary tumor and spread to other parts of the body via either the lymphatic system or the blood stream (Lee et al, 1986), establishing new colonies and ultimately a new tumor. This process by which cancer spreads from an original site to form tumors at distant sites is known as metastasis. It is these secondary tumors that produce the destructive damage that we associate with the disease. Such secondary deposits arising from a cancer elsewhere in the body are extremely rare varieties of breast cancer. The breast is frequently the site of primary tumors. Alternatively, organs such as the liver are seldom the site of primary tumors, but are often involved with a secondary process. Yet other organs, like the lungs, are frequently the site of both. Distant metastases are seen most commonly in the bones, lung, pleura, liver, adrenal gland, and central nervous system (Cifuentes and Pickren, 1979).

Often carcinoma of the breast will metastasize through the veins to the lungs, vertebral bodies, skull, pelvic bones, and central nervous system (Haagensen, 1986; Hendriques, 1962). The lymphatic system removes waste products and delivers them into lymph nodes. These nodes contain highly specialized cells called lymphocytes which attack and

destroy breast-cancer cells. If these metastatic cells overwhelm the defence mechanism of the lymph nodes, cancer cells may spread to other parts of the body (Pickren, 1956).

Approximately half of all breast cancers arise in the upper, outside quadrant of the breast, which may simply be because it has more breast tissue. The location, however, in which the cancer starts does not seem to affect the treatment or prognosis. Pathologists consider three other factors more important in determining a cancer's stage and treatment: the extent or size of the primary tumor; the degree of spread to lymph nodes; and the presence of metastases to other parts of the body (Alexieva-Figusch et al, 1988).

Epidemiology

Cancer of the breast is the most common cancer among North American women, and has been so since 1940 when the lifetime risk was one-in-twenty (NCI Data, 1992). The incidence of the second and third most common cancers in 1940, cancer of the uterus and cancer of the stomach, have since decreased by as much as seventy percent in the 1990s. In comparison, the incidence of breast cancer has increased by eighty percent. The lifetime risk of getting breast cancer is now one-in-nine for women who live to be eighty-five, and one-in-eight for women who live longer. The number of new cases diagnosed between 1940 and 1982 had been rising at an annual rate of 1.1 percent, and has since drastically increased by quadrupling to 4.3 percent a year (Miller et al, 1991).

Breast cancer is most common among women over the age of fifty. At least some of the increase in incidence and risk is due simply to a thirteen year increase in the life expectancy for women in North America over the last thirty years. The number of women older than forty-four has increased sixty-four percent between 1960 and 1990. The dramatic increase in breast cancer incidence in the 1980s can mostly be attributed to greater efficiency in early detection. With increased screening, breast cancer is being diagnosed at more treatable, early stages of growth while lesions are in the "*in situ*" stage. Even after adjusting the statistics to compensate for an older population and for early

detection, there is still an upward swing in the incidence of breast cancer (Glass and Hoover, 1990; Liff et al, 1991).

If the increase in breast cancer in the eighties is indeed mostly the consequence of early detection, there should be little or no impact on breast cancer incidence in the long run. We should return to the unsettling one percent steady increase, which remains to be accounted for, by the mid-1990's. Early detection, however, should accompany a decrease in mortality rates in the future, which has remained constant over the past twenty years (Kessler et al, 1991). Until 1985, it was the leading cancer killer among North American women, a distinction now claimed by lung cancer (Silverberg and Lubera, 1988). Experts have suggested a wide variety of reasons to explain this annual one percent increase in the incidence of breast cancer.

In a minority of cases (about five percent of all breast cancers) there is evidence clearly in favour of a familial-heredity role in the development of breast cancer, which increases the risk two to three times for an individual (Anderson, 1972 and 1974; Sattin et al, 1985). It has been consistently observed that women have a higher risk of developing breast cancer if they have a strong family history of the disease. Some investigators have observed this familial risk to be greater if the index case is premenopausal (Anderson, 1972; Sattin et al, 1985). These results are consistent with data attained by Mary-Clair King on a breast cancer susceptibility gene, on the long arm of chromosome 17 (BRCA1), which is linked with families with early-onset breast cancer (Hall et al, 1990). Since these rare, high risk cancers account for only five percent of all breast cancers, there is no significant impact on the overall incidence of breast cancer in the general population.

Epidemiologists in the 1980s theorized that eating fatty foods may be linked with an increased risk of breast cancer (Armstrong and Doll, 1975; Hirayama, 1978; Miller et al, 1978), which would explain why North American and European women have a five times greater risk of acquiring the disease than women in Asia and Africa (Silverberg and

Lubera, 1988; Parkin et al, 1988). Japanese migrating from a low risk environment to a high risk environment like North America gradually increase their risk over two to three generations, demonstrating that something in the environment stimulates the disease (Dunn, 1977). Conversely, studies of meat-abstaining nuns (Kinlen, 1980) and lactoovovegetarian Seventh-Day Adventists (Phillips et al, 1980) have found no association with animal fat or meat intake. Walter Willett, along with other investigators, also dispute the circumstantial evidence implying an association between a fatty diet lifestyle and breast cancer (Willett and Stampfer, 1990; Willett et al, 1987; Jones et al, 1987). Walter Willett used data from one of the most thorough and exhaustive studies implemented, involving 120 000 women over several years. It was launched in 1976 and is known as the Harvard Nurses Health Study. He once again found no association between high fat diet and breast cancer, and in addition claims that women on an extremely high fat diet and women on an extremely lean diet have the same risk of developing breast cancer (Harris et al, 1992).

The direction of epidemiological research is once again refocussing on a previously suspected culprit: hormones, especially a longer exposure to estrogen.

Mitogenic Regulation: Hormones and Growth Factors

It has been established that hormones affect all body organ systems, and are critical for the control of normal sexual development and reproduction. Hormones (from the Greek word *hormon*, which means "to excite") are produced by glands in the endocrine system which secrete their hormones directly into the bloodstream. The hormones travel through the circulation, bind to their specific receptors, and set in motion the desired responses in the organ where their receptors are present. As noted earlier, the breasts are particularly sensitive to hormonal influences. A substantial body of experimental, clinical, and epidemiological evidence indicates that the same hormones play a major role in the etiology of breast cancer (Henderson et al, 1988a; 1988b).

Estrogen is an appropriate focus for the discussion of the hormonal causes of breast cancer, since, for a century now, the epidemiology of breast cancer seems to implicate the secretion of ovarian estrogen in the neoplastic process. Glasgow surgeon Sir George Beatson reported to the British medical journal *Lancet* in 1896, a series of operations in which women with breast cancer were successfully treated by bilateral ovariectomy. "Eight months after castration," he wrote, "all vestiges of [the breast] cancer disappeared." (Beatson, 1896). Half a century later, Huggins and Bergenstal used hormone removal, by introducing surgical adrenalectomy, as a treatment for breast cancer in postmenopausal women. Recent studies have shown that patients with premature ovarian failure rarely get breast cancer (Poortman, 1980). Foremost is the observation that breast cancer is one hundred times more common in women than men even though men also have breast tissue, but without the lobules found in female breasts.

Other risk factors for the development of breast cancer in women include: first, an early age of menarche, which has progressively decreased in North Amercia due to control of infectious diseases and improved nutrition. Attainment of a critical body weight/height ratio appears necessary for menarche to occur (Frisch and McArthur, 1974). This may explain geographical/ethnic differences in breast cancer incidence observed in North America, China (Marshall et al, 1992) and Japan (Pike et al, 1981). Second, with a late menopause, for example at age 55 the risk of breast cancer doubles compared to those who experience menopause at age 45 (Trichopoulos et al, 1972).

A third factor is excess weight. Obese patients have an increased storage of lipids and decreased sex hormone binding globulin, which increases the unbound fraction of estrogen (Ingram et al, 1989). As a result, an increase in weight after age 50 by a 10kg increment increases breast cancer risk by 80% (de Waard et al, 1977).

Finally exogenous estrogen, such as estrogen replacement therapy for postmenopausal women and the use of oral contraceptives have been linked to increased breast cancer incidence (Key and Pike, 1988; Dupont and Page, 1991). Other studies, however, did not

find any association (Steinberg et al, 1991). Further analysis is required to resolve this controversy regarding exogenous estrogen and the rusk of breast cancer.

Rodent models have been widely used to study the influence of estrogen on breast cancer. Investigators have examined whether estrogen can act to: initiate malignant cell transformation of mammary cells as a true carcinogen; promote tumor cell proliferation and progression; and indirectly initiate neoplastic transformation, taking into consideration the abundant growth factor production and somatic mutations observed in cells when they become neoplastic (Davidson and Lippman, 1989).

Prolonged administration of estrogen by injection, or as Dunning and coworkers demonstrated by implantation of estrogen (diethylstilbestrol (DES) pellets, resulted in the induction of mammary cancer in certain strains of inbred rats (Dunning et al, 1947). Others confirmed these findings, reporting that carcinomas that were estrone dependent would regress when the exogenous source of estrone was removed, and could be revived by readministering an estrogen source. Despite these progressive findings, the data does not demonstrate unequivocally that estrogen is the sole cause of mammary cancer.

Important laboratory advances have been made using animal models. This model system displays some features observed in mammary malignancies: a long latent period, metastases, and hormonal dependence of the tumor (Cutts and Noble, 1964). However, these models do not mimic breast cancer in clinical patients. Rather, they seek to understand the process of malignant transformation with the hope that important information can be extrapolated to aid in the treatment of patients.

The complete evaluation of the endocrinology of breast cancer is no longer confined to the effects of serum estrogen levels in mammary tissue. Much higher concentrations of estrogen are observed in the tissue environment, thereby shifting the emphasis in endocrine research from measurements of blood levels to examination of local factors that control hormone levels within the breast. Tissue concentrations of steroid hormones are

determined by glandular secretions found in serum, synthesis of active steroids from precursor compounds, as well as degradation or detoxification within the target organ to less active hormone derivatives. Compared to normal breast tissue, malignant breast tissue has elevated levels of aromatase activity, the enzyme that converts androgens to estrogen, and as a result have enhanced ability to synthesize estrogen from precursor steroids (Santen et al, 1986; James et al, 1987). Some investigators have suggested that epidermal growth factor (EGF) may contribute to aromatase activity (James et al, 1987), as well as have direct effects on tumor growth. The synthetic degradative steroid pathway is also altered to favor tumor growth. The conversion of estrodiol to a more highly estrogenic derivative, 16 α-hydroxyestrone and estriol, by 16 α-hydroxylation is amplified (Bradlow et al, 1986), whereas, the metabolism of estrodiol to less estrogenic steroids, such as estrone by the enzyme 17 β-hydroxysteroid dehydrogenase, is suppressed (Bonney et al, 1986; Vermuelen et al, 1986; Ernster et al, 1987).

The lipid soluble estrogen molecule begins its biochemical mechanism of regulating cell growth by diffusing across the cell membrane and binding to specific high-affinity receptor proteins within the cell. Work on the high-affinity estrogen binding process was pioneeered by Jensen in the 1950's who demonstrated long-term retention of estrogen by the rodent uterus (Jensen, 1958; Jensen and Desombre, 1972). The binding component is now recognized as the estrogen receptor. Unbound ER resides in the nucleus (Zava and McGuire, 1977; King and Greene, 1984; Welshons et al, 1984) and previously detected cytoplasmic ER (Jensen and Desombre, 1972; Edwards et al, 1980a) is now recognized as an artifact of tissue processing (King et al, 1985). The estrogen-ER complex dimerizes on the hormone response element present on the target gene, then foms a complex with the nuclear matrix (Barrack and Coffey, 1980) and alters its confirmation to promote specific gene transcription (Klein-Hitpass et al, 1989; Strobl and Thompson, 1985; Pardoll et al, 1980; Robinson et al, 1985). The different regions or domains of the ER and their functional significances, along with interactions with nuclear components, have been extensively researched, but will not be addressed here.

Physiological concentrations of estrogen have been shown to stimulate the growth of estrogen responsive cell lines in culture by many laboratories (Soule et al, 1973; Lippman et al, 1976; Engel et al, 1978; Engle and Young, 1978; Weichselbaum et al, 1978; Chablos et al, 1982; Leung et al, 1982; Page et al, 1983; Darbre et al, 1983; Natoli et al, 1983; Simon et al, 1984; Whitehead et al, 1984; Berthois et al, 1986; Reiner and Katzenellenbogen, 1986; Dubik et al, 1987; Dubik and Shiu, 1988). Cell culture systems have contributed greatly to our understanding of the growth regulation of breast cancer cells. Examining specific populations of cells under defined conditions has distinct advantages; however, several limitations must be considered. The select population (or subpopulation) of cells able to adapt and grow under defined conditions in suspension or on plastic striatum, are not representative of the malignant cells in a primary tumor. Observations may very vastly from one laboratory to another if different conditions are used (over many years) and ultimately may no longer be relevant to our understanding of tumorigenesis *in vivo*. Lastly, tissue culture systems do not address the complex interactions between tumor epithelial cells and surrounding stromal and vascular elements found *in vivo*. Despite the considerations, numerous studies using *in vitro* (as well as *in vivo*) model systems have enabled us to unravel the complex mechanism of the growth regulation of breast cancer cells.

A number of human breast cancer cell lines exist and have been extensively characterized. Several estrogen receptor positive/estrogen responsive cell lines, MCF-7, T-47D, and ZR-75-1 (Soule et al, 1973; Keydar et al, 1979; Engel et al, 1978) generally grow poorly, if at all, without estrogen supplementation in culture or in ovariectomized nude mice. In contrast, estrogen receptor negative/estrogen unresponsive cell lines, such as MDA-MB-175, MDA-MB-231 and SK-BR-3, grow readily without estrogen.

Estrogen treatment of human breast cancer cells results in a variety of biological responses (Lippman et al, 1977). The activity of several regulatory enzymes, including those necessary for nucleic acid synthesis, such as thymidine kinase, dihydrofolate reductase, and DNA polymerase, are induced by estrogen (Edwards et al, 1980b; Aitken and

Lippman, 1983, 1985; Cowan et al, 1982; Kasid et al 1986). Estrogen treatment results in increases in thymidine incorporation and DNA synthesis (Aitken and Lippman, 1983), along with transcription of estrogen regulated genes directly associated with growth (Aitken et al, 1985). Despite these findings, controversy remains concerning the mechanism by which estrogen after binding to the estrogen receptor, stimulates the growth of breast cancer cells.

The direct effect of estrogen on the growth of cultured cells has been inconsistent (Lippman et al, 1976; Edwards et al, 1980b, Shafie, 1980; Soto and Sonnenschein, 1985), leading to the speculation that estrogen may act indirectly through an intermediate growth stimulator (Sirbasku, 1978) or by blocking certain growth inhibitory factors found in serum (Soto and Sennenschein, 1985). Estrogen was shown to stimulate a "second messenger" regulatory system during the estrogen stimulated growth of MCF-7 cells. Phosphatidylinositol (PI) turnover was observed twelve to twenty-four hours after estrogen treatment (Freter et al, 1988). Others have observed similar PI turnover by growth factors with a much more rapid stimulation, occurring within minutes (Nishizuka, 1984). This delayed effect of estrogen on PI turnover is consistent with an intermediate induction of autocrine growth factors by estrogen, which then stimulates PI turnover. Autocrine loops have also been observed in normal circumstances, which are closely controlled by either internal programming of the cell or interruption by an external stimuli. Since the proposal of autocrine or self-stimulating polypeptide growth factors, a tremendous amount of work has been directed towards understanding the estrogen regulation of growth factor secretions and their effect on cancer cell growth.

The processes of cellular proliferation and differentiation are essential features of the successful development and replenishment of tissues, and in multicellular organisms shows a remarkable degree of coordination with the surrounding tissue environment. The importance of intercellular communication was demonstrated seventy years ago when the developmental fate of *Xenopus* embryos was redirected by the adjacent implantation of tissue originating from other regions (Spemann and Mangold, 1924). Although physical

parameters, extracellular matrix components, and cell adhesion molecules influence developmental processes, a critical role is played by soluble factors. The growth promoting properties of serum and tissue extracts are the foundation from which the science of tissue culture was developed. It was the fractionation of these complex solutions that originally led to the discovery of nerve growth factor (NGF) (Levi-Montalcini, 1987) and epidermal growth factor (EGF) (Cohen, 1986). Subsequently, these discoveries led to the identification of a wide array of secreted polypeptide growth factors that act as positive and negative modulators of cellular growth in virtually all cells, and influence differentiation.

A variety of hormones, neurotransmitters, and growth factors which are too large to pass through the cell membrane, exert their effects by interacting with receptor proteins on the surface of target cells. Specific high affinity interaction of the signal molecule, or ligand, with its corresponding cell surface receptor triggers one or more intracellular signals that alters the behaviour of the target cell. By using receptor-binding ligands labelled with radioactive atoms, fluorescent dyes or electron dense particles, it has been shown that the distribution of cell surface receptors for a specific ligand can be either diffuse or localized to specific regions of the plasma membrane and that their numbers vary depending on the receptor. Techniques for cloning the DNA sequences encoding cell surface receptors have revolutionized our understanding of receptor structure and function. Unlike nuclear receptors for steroids, vitamin D3 and thyroid hormones, cell surface receptors do not regulate gene expression directly. Instead, ligand binding forces an appropriate conformational change in the cell surface receptor protein that relays a signal across the plasma membrane, before the receptor-ligand complex is rapidly internalized and degraded (Freissmuth et al, 1989 for review).

Studies of immediate events that accompany ligand-receptor binding suggest a common sequence of events for all receptors with cytoplasmic tyrosine kinase domains, such as epidermal growth factor, insulin, IGF-I, and PDGF receptors (Cantley et al, 1991; Ullrich and Schlessinger, 1990). Ligand-induced activation of the kinase domain, and its signal

potential, are mediated by receptor oligomerization. Ligand binding and the subsequent conformational alterations of the extracellular domain induce receptor oligomerization, which stabilizes interactions between adjacent cytoplasmic domains and leads to activation of kinase function by molecular interactions. As a result, transmission of the conformational change from the extracellular domain to the cytoplasmic domain occurs without requiring alterations in the positioning of amino acid residues within the transmembrane domain. Receptor oligomerization is a universal phenomena among growth factors, and results in elevated protein tyrosine kinase activity and enhanced ligand binding affinity (Czech, 1989; Ullrich and Schlessinger, 1990).

Most cell surface receptor proteins belong to one of three classes which are defined by the transduction mechanism used (Berridge, 1985 for review). (1) Receptors of the channel linked class are transmitter gated ion channels and are mainly involved in rapid synaptic signalling between electrically excitable cells. This type of signalling is mediated by a small number of neurotransmitters that transiently open or close the ion channel to which they bind, briefly changing the ion permeability of the plasma membrane, and therefore, the excitability of the postsynaptic cell. DNA sequencing studies have shown that channel linked receptors belong to a family of homologous transmembrane proteins which span the plasma membrane more than once. (2) Receptors of the catalytic class operate directly as enzymes when activated by their ligand. Almost all the known catalytic receptors are transmembrane receptors with a cytoplasmic domain that functions as a tyrosine-specific protein kinase. (3) Receptors of the G protein linked class indirectly activate or inactivate a separate plasma membrane bound enzyme or ion channel. The interaction between the receptor and the enzyme or ion channel is mediated by a third protein, called a GTP-binding regulatory protein, or G protein. The G protein linked receptors usually activate a cascade of events that alters the concentration of one or more small intracellular signalling molecules, often referred to as intracellular messengers (Birnbaumer, 1990). These intracellular messengers act in turn to alter the behaviour of yet other target proteins in the cell.

Two important G protein regulated intracellular pathways involve cyclic AMP synthesis from ATP by the plasma membrane bound enzyme, adenylate cyclase, and the hydrolysis of phosphatidyl inositol 4,5-biphosphate (PIP_2) by phospholipase C (PLC). PLC activity yields the second messengers 1,2-diacylglycerol (DAG), which activates protein kinase C and inositol 1,4,5- triphosphate (IP_3) which mobilizes Ca^{2+} from intracellular stores (Michell, 1992). One potential target for protein kinase C is the increase in ion flux across the plasma membrane (Na^+/H^+ antiporter), which is activated in a number of mitogen-triggered proliferation systems resulting in cytoplasmic alkalinization. Protein kinase C is not an oncogene; however, its expression in fibroblasts has been observed to lead to disordered morphology (Housley et al, 1988; Persons et al, 1988).

Since the discovery of cyclic AMP (cAMP) by Sutherland and coworkers (Rall and Sutherland, 1958), the hormone regulated adenylate cyclase system has been studied as one of the principal mediators for transmembrane signalling pathways. Birnbaumer and coworkers (Rodbell et al, 1971a, 1971b) achieved a major breakthrough when they demonstrated that both hormones and GTP are required for stimulation of adenylate cyclase activity. Further investigation provided the first evidence that the transmembrane signalling pathway was composed of at least three distinct proteins: a receptor, a catalyst, and an intermediary transducer. The transducer protein was purified and shown to be a guanine nucleotide binding protein that in its GTP-bound state stimulated adenylate cyclase; thus, it was termed G_s (s=stimulatory). The subsequent purification of G_i, the G protein necessary for receptor mediated inhibition of adenylate cyclase, explained the bidirectional regulation of this enzyme activity (Bokoch et al, 1983). However, the identification by Sternweis and Robishaw (1984) and independently by Neer and coworkers (1984) of G_0 for which no obvious function was evident, indicated that G proteins served a broad role in linking plasma membrane bound receptors to membrane bound effectors.

Considerable research has focussed on elucidating the action of cAMP which has been found to act as an allosteric effector, activating specific proteins by binding to them and

changing their conformation. Cyclic AMP exerts its effects in eukaryotic cells mainly by activating an enzyme called cAMP dependent protein kinase A (PKA) which controls many biochemical events by catalyzing the transfer of the terminal phosphate group from ATP to specific serine and threonine residues of selected proteins in the target cell (Van Buskirk et al, 1985). Covalent phosphorylation of the appropriate residues regulates the activity of the protein and this protein in turn regulates other proteins. The mechanism by which an increase in cAMP activates the transcription of specific genes still remains to be determined.

In some cells, an increase in cAMP activates the transcription of specific genes. In neuroendocrine cells of the hypothalamus, cAMP causes activation of the gene that encodes the peptide hormone somatostatin (Montminy and Bilezikjian, 1987). The promoter region of the somatostatin gene contains a short DNA sequence (~30 basepairs) that is also found in the promoter region of several other genes that are activated by cAMP. This sequence is recognized by a specific 43 kDa cAMP response element binding protein (CREB) that activates transcription from these genes when phosphorylated. However, this mechanism only applies to genes which have the cAMP response element (CRE) and does not explain the increase in transcription of other genes which do not contain a CRE. The recent observation that the neurotransmitter, dopamine, which binds to its cell surface receptor and results in activation of a member of the nuclear hormone receptor family causing transcriptional activation of some genes, may provide insight into other potential cAMP mediated transcription mechanisms.

The production of autocrine growth factors by human breast cancer cells as modulators of cell growth was supported by observations that the initial growth rate of MCF-7 cells in culture was directly proportional to the number of cells plated. In addition, deactivation of conditioned medium with trypsin, the reducing agent dithiothreitol, or 56°C heat markedly reduced its ability to support tumor growth; suggesting that a polypeptide factor(s) was responsible for growth promotion.

Several growth factors including TGF-α, IGF-I, IGF-II, PDGF, and the epithelial cell inhibitor TGF-β, are expressed by breast cancer cells (and tissue) and secreted into the surrounding medium of cultured breast cancer cells. In addition, a variety of receptors for insulin, IGF-I, IGF-II, EGF, and TGF-β have been identified in cultured breast cancer cells or in human breast cancer biopsy specimens. Therefore, serum growth factors may potentially stimulate their own growth in an autocrine fashion (TGF-α or IGF-II) or could affect stromal tissue (TGF-β or PDGF) or induce tumor cell invasion by paracrine mechanisms. TGF-β1 stimulates the growth of fibroblasts while inhibiting the growth of most epithelial cells including cancer cells.

More intriguing is the observed effect of estrogen on growth factor transcription. The synthesis and secretion of several of the above growth factors are regulated by estrogen in some estrogen receptor-positive cells, and are produced constitutively by certain receptor-negative cells (Lippman et al, 1987). TGF-α and IGF-II are increased by estrogen, whereas the reverse is observed with TGF-β, a potential tumor inhibitor (Tucker et al, 1984; Roberts et al, 1985). TGF-β secretion is induced by antiestrogens in human breast cancer cells *in vitro* and inhibits the growth of estrogen receptor-positive and -negative breast cancer cells *in vitro* (Roberts et al, 1985; Ranchalis et al, 1987; Knabbe et al, 1987; Arteaga et al, 1988b; Zugmaier et al, 1989). Loss of estrogenic control of breast cancer growth during malignant progression lead to the speculation that the growth advantage associated with estrogen receptor-negative tumors may be due to the constitutive production of autocrine growth factors.

The presence of a variety of growth factor receptors on cell surfaces suggests that cell development and proliferation *in vivo* are determined by combinations of interacting stimuli. Rodent fibroblasts were analyzed *in vitro* to identify "restriction points" in their cell cycle. It was determined that competence or "early response" factors such as PDGF causes quiescent (G0) cells to advance into the preliminary (G1) phase of the cell cycle. PDGF also sequentially induces the proto-oncogenes *c-fos* and *c-myc* in the nucleus, and

c-ras found in the plasma membrane (Heldin and Westermark, 1984). Epidermal growth factor (EGF) or TGF-α acts later, and IGF-I still later in G1 as progression factors which commit the cell to the DNA synthesis phase of the cell cycle (Stiles et al, 1979). Both EGF and IGF-I are required to trigger the essential switch, as the two factors stimulate complimentary components of the signal transduction network (Rozengurt, 1986). The loss of requirement for exogenous serum growth factors by some cancer cells and by cells transformed by various tumor viruses, oncogenes, chemicals or radiation is due to self-production of their necessary growth factors, thereby, allowing them to continue to proliferate by bypassing restriction points in the cell cycle (Cherington et al, 1979; Bradshaw and Dubes, 1984; Heldin and Westermark, 1984; Zhan and Goldfarb, 1986).

The uncoupling of the interdependent processes of proliferation and differentiation in normal cell development is an essential step in the generation of the transformed phenotype, and hence of cancer. The ectopic production of growth factors, such as PDGF (*c-sis*), EGF or TGF-α, TGF-β, IGF-I, IGF-II, FGF-1, -2, -3 (*int-2*), -4, -5, have also been shown to contribute significantly to the process of transformation. Expression vector mediated production of an autocrine growth factor in growth factor-dependent hemopoietic and fibroblast cell lines results in the formation of factor independent and tumorigenic cells. There is reason to believe that these transforming effects are not achieved entirely by a classical autocrine pathway, whereby the growth factor is secreted into the extra-cellular medium before binding and stimulating cell surface receptors. First, the strong transforming stimulus through the overexpression of *c-sis* (Josephs et al, 1984) is not reproducible by addition of exogenous PDGF (Bejcek et al, 1989). Second, both the extracellular and intracellular presence of FGF-1 or FGF-2, neither of which has a recognizable signal sequence, is required for the transformation of fibroblasts. These observations are consistent with a mechanism by which premature binding of surface receptors occurs in the internal membranes of the endoplasmic reticulum and the golgi apparatus. This intracrine hypothesis has received much attention recently since the potential to exert mutually exclusive effects on the expressing cell, compared with those on neighbouring cells, is wide ranging. Perhaps intracellular ligand binding is not subject

to rapid receptor downregulation resulting in overstimulation of the intracellular control processes which would constitute a strong transforming signal. This may be the case since EGF (*erb B*), M-CSF (*fms*), and SLF (*kit*) are constitutivly activated receptors, independent of ligand binding, and have strong oncogenic potentials (Downward et al, 1984; Sherr et al, 1985; Chabot et al, 1988; Geissler et al, 1988). The discovery that many oncogene products are altered forms of growth factors or growth factor receptors provided further evidence for the importance of a defective mechanism of hormonal control in cancer.

Following uncoupling of normal systems controlling proliferation and differentiation, the next step in tumorigenesis or tumor evolution is extensive growth of the transformed cells. The oncogenic potential of *sis* and *int-2 in vivo* suggests a decisive involvement in the early stages of tumorigenesis. Although as mentioned earlier, transforming abilities of many growth factors have been observed *in vitro*, a more likely contribution *in vivo* is to the later stages of tumor progression. That is, after the transformation event has taken place. Establishment of an autocrine growth response results in less dependence on environmental factors provided by other cells.

As noted earlier, the control of tumor growth is not limited to malignant cells alone, but rather depends also on an intimate and complex interrelationhip with surrounding non-tumor tissue. Paracrine acting growth factors and oncogenes, such as *c-sis*, may be intercellular mediators complementing tumor growth (Sporn and Roberts, 1985). After analyzing benign and malignant biopsy tissue, Lippman and coworkers proposed a cooperative paracrine loop between malignant cancer cells and fibroblasts, commonly observed surrounding breast cancer tissue. The secretion of PDGF by tumor cells stimulates stromal proliferation and the release of IGF-II from surrounding fibroblasts, which acts back on the tumor to induce growth (Cullen et al, 1991).

At some point in their growth, tumor cells secrete angiogenic proteins in order to stimulate vascular growth, a process known as angiogenesis. Thus far, four angiogenic

proteins have been found to be produced by cultured breast cancer cells: basic fibroblast growth factor, vascular-endothelial growth factor, pleiotrophin, and platelet-derived endothelial cell growth factor. In the absence of vascularization, nutrients no longer reach the center of the spherical tumor and proliferation of cells at the tumor surface is balanced by cell death in the center. Since tumors need their own blood supply to grow beyond a 2 to 3 millimeter diameter, vascularization removes the limitations of diffusion through solid tissues and the tumor can then rapidly increase in size. Most small, nonvascularized tumors have long been known to be unable to metastasize. Knowing this, Oxford researcher Adrian Harris hypothesized that blood vessels may provide the opening through which metastatic cells can spread to distant sites in the body. Several reports have confirmed this hypothesis, stating that the extent of angiogenesis predicted the occurrence of metastasis better than other indicators, and is now being used as a prognostic indicator. Anti-angiogenesis compounds that block the activities of angiogenesis-stimulating proteins are now being tested as therapeutic agents for breast cancer.

B. The Insulin-like Growth Factor-II Protein

The originally proposed one growth factor-one receptor model is no longer accepted. For every growth factor system there exists a complex family of growth factors that bind to multiple receptors, and are regulated by serum binding proteins. The insulin-like growth factors are no exception. Their complex regulation occurs at the transcription level, during post-transcriptional processing, in serum by regulatory binding proteins, and when binding to functionally distinct receptors on the cell surface. A tremendous amount of research over the past decade has generated an explosion of knowledge concerning the IGF's, which has resulted in this family of molecules being the best characterized of the peptide growth factors to date.

Historical Background

The discovery of the IGF's arose from three areas of research, identifying three distinct biological activities in serum: (i) plasma intermediaries of growth hormone action; (ii) peptides in plasma with insulin-like metabolic actions; and (iii) autocrine factors that sufficiently maintain growth of various cells in the absence of serum.

Original observations of an intermediary peptide mediating growth hormone actions were made by Salmon and Daughaday (1957). They measured [^{35}S]-sulfate incorporation into cartilage as an *in vitro* bioassay. They, and others, had inconsistent results when they attempted to stimulate sulfate uptake in hypophysectomized rat cartilage *in vitro* with GH. However, these authors demonstrated that serum from normal rats, but not from hypophysectomized rats, could correct the defect in the synthesis of matrix proteins (sulfate uptake). This intermediary substance in serum was operationally defined as "sulfation factor", and later "somatomedin" by McConaghey and Sledge (1970). The subsequent isolation and sequencing of somatomedin C (Klapper et al, 1983) and A (Enberg et al, 1984) has identified them as IGF-I, and the designations somatomedin and IGF-I are used synonymously. The accelerated growth of hypophysectomized rats *in vivo* receiving recombinant IGF-I greatly strengthens the somatomedin hypothesis (Schoenle et al, 1982).

During this same early period, specific antibodies were used to measure plasma insulin levels (Slater et al, 1961; Leonards et al, 1962). It became apparent, with the development of the radioimmunoassay for insulin, that serum contains far more insulin-like activity than could be accounted for by immunoreactivity. This insulin-like activity was termed non-suppressible insulin-like activity (NSILA) (Froesch et al, 1963). The Zurich team of Rinderknecht and Humbel (1976, 1978) purified two similar but distinct peptides from an acid ethanol extract of plasma (Cohan fraction of human plasma), then sequence analyzed the two peptides. Because of their structural and functional similarity to insulin, they were called insulin-like growth factors I and II.

Temins suggested that NSILA was the essential factor in serum with multiplication-stimulating activity (MSA). He later purified NSILA from calf serum and showed that it was a potent stimulator of cell growth in culture. Dulak and Temin (1973) suggested that cells able to grow in the absence of serum produced a multiplication-stimulating factor. They isolated a peptide with the properties of a somatomedin from a serum independent rat hypatocyte cell line (BRL-3A). The final purification was achieved by Marquardt et al (1981) and its amino acid sequence was shown to be nearly identical to human IGF-II. This was the first evidence that somatomedins could act locally as autocrine and paracrine factors.

Gene Structure and mRNA Transcripts

Human IGF-II is the product of a single gene locus mapped on the distal short arm of chromosome 11 (11p15) spanning 30 kb of chromosomal DNA (Brissenden et al, 1984; Tricoli et al, 1984). The gene is only 1.4 kb downstream and contiguous with the insulin gene (Bell et al, 1985), which is found just 1.5 kb downstream, and contiguous with the gene for tyrosine hydroxylase (O'Malley and Rotwein, 1988). Despite their close physical association on the chromosome, these genes are expressed in different tissues and at different developmental stages. The organization of mouse (Stempien et al, 1986) and rat (Dull et al, 1984; Soares et al, 1985; Ueno et al, 1987) genes also form a conserved linkage group with insulin either on chromosome 1 in the rat (Frunzio et al, 1986; Soares et al, 1986) or chromosome 7 in the mouse (Brilliant et al, 1987). It has been suggested that gene duplication lead to the divergence of proinsulin and a primitive IGF gene more than 600 million years ago before the appearance of vertebrates. A second duplication gave rise to IGF-I and IGF-II about 300 million years ago with the appearance of mammals (Blundell and Humbel, 1980). [The human and rat genes have multiple promoters, highly homologous coding regions, long 3' untranslated regions, dual polyadenylation sites, and conserved positioning of introns within exons E1 to E6.]

Several structural motifs are shared among IGF-II genes from different species, beginning

with multiple 5' untranslated exons in humans, rat and mouse genes. Human and rat IGF-II gene structures are depicted in Figure 1. The coding region for the prepro-IGF-II precursor, consisting of a signal peptide of 24 amino acid residues, the 67 residues of the growth factor, and of an E peptide 89 residues long, is found within the last three exons (E4, E5, and E6). The first two of these exons encode the 24 residue signal peptide, the mature 67 residue IGF-II peptide, and the initial 11 amino acids of the E domain. The remaining part of the E domain and the 3' untranslated region of the IGF-II mRNA are found on the last exons. Four different promoter regions have been identified using primer extension and nuclease S1-protection analysis in the human gene upstream of exons E1', E1, E2, E3 and three promoters are present in the rodent upstream of exons E1, E2, and E3 (Ueno et al, 1987, 1989; Holthuizen et al, 1990). Transcriptional initiation from the different promoters give rise to IGF-II mRNAs with different 5' ends and provide a mechanism for differentially regulated gene expression. Complex gene regulation is synonymous with growth factors whose actions are multifaceted.

The IGF-II gene is expressed in multiple tissues and organs, but all studies indicate a far more abundant expression during fetal development than in the adult. Multiple sized transcripts are present in fetal tissue due to differential initiation of transcription occuring at distinct promoter sites (P1, P2, and P3; de Pagter-Holthuizen et al, 1988). Transcription from these promoters show a developmental and tissue-specific mechanism of regulation (de Pagter-Holthuizen et al, 1987). The most abundent transcripts are the 6.0 and 2.2 kb species derived from promoter P2. Transcripts of 5.0 and 4.8 kbs derived from promoters P1 and P3, respectively, have also been described but are less abundant. Promoters P1 and P3 appear to be less active, although it is possible that these transcripts are less stable.

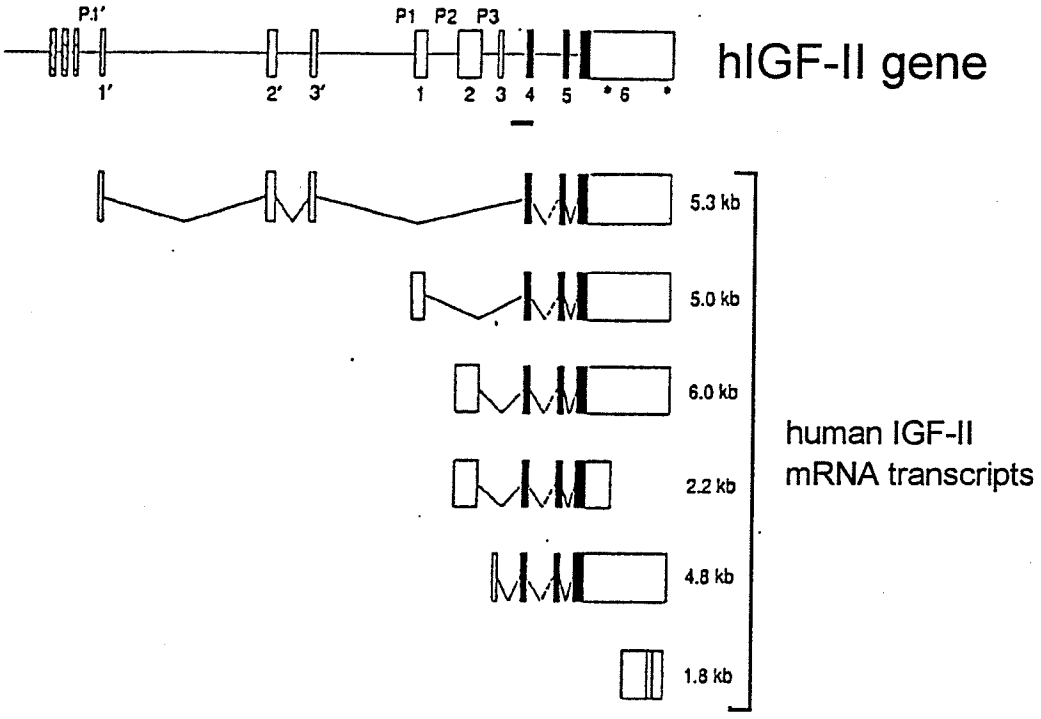
Expression of tissue IGF-II mRNA is highest during fetal development and declines during the postnatal period, with the notable exception of the liver, although the mechanisms of attenuation are unknown (Gray et al, 1987). In most postnatal human tissues promoters P1, P2 and P3 retain a low level of activity, with the 4.8 kb transcript

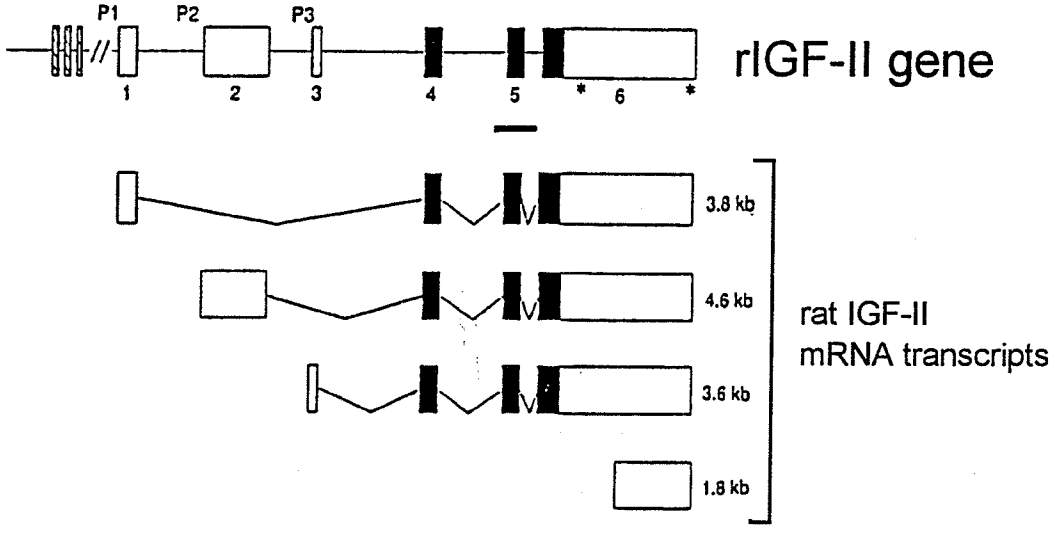
Fig. 1 ⇒

both sides

Figure 1. Structure of human and rat IGF-II genes and their corresponding mRNAs.

The numbered boxes indicate the exons (E1'-E6) of the IGF-II genes. The location of the promoters (P1'-P3) and polyadenylation signals (*) are labelled accordingly. Horizontal bars represent a distance of approximately 1kb in respective genes. Below the genes are IGF-II mRNA transcripts derived from multiple promoters and poly A addition signals. Solid black boxes represent regions coding for the IGF-II preproIGF-II precursor protein while non-coding regions are in white. Transcripts also describe promoter usage, splicing, and polyadenylation site used in different IGF-II mRNA species. Broken zigzag lines between exon 4 and 5 in the human gene indicates alternative splicing associated with exon 5. The resulting transcript sizes are given to their right. The three boxes prior to the IGF-II genes encode for insulin in respective species.


P1' P1 P2 P3
1' 2' 3' 1 2 3 4 5 6
hIGF-II gene
5.3 kb
5.0 kb
6.0 kb
2.2 kb
4.8 kb
1.8 kb
human IGF-II
mRNA transcripts


P1 P2 P3
1 2 3 4 5 6
rIGF-II gene
3.8 kb
4.6 kb
3.6 kb
1.8 kb
rat IGF-II
mRNA transcripts

derived from promoter P3 perhaps being the prevailing one. An exception is the liver, where there is a developmentally regulated switch to the exclusive use of promoter P1', resulting in the characteristic 5.3 kb mRNA transcripts (Bell et al, 1985; Gray et al, 1987; Irminger et al, 1987; de Pagter-Holthuizen et al, 1989). No such change occurs in mRNA or promoter type in other organs (Bell et al, 1985). Promoter P1' is only expressed in adult human liver and no analogous promoter has been demonstrated in the rodent IGF-II gene. Its absence may explain the sharp decrease in serum IGF-II after birth in rats (Moses et al, 1980), but not in humans (Ashton et al, 1985) or in pigs who have a P1' counterpart in their genome (Hedley et al, 1989).

The first physiological significance for IGF-II in fetal development was the consequence of disrupting the IGF-II gene by homologous recombination in the mouse. Successful targets were 60% of normal body weight but normally proportioned. The smaller mice were the result of germ-line transmission of the inactivated IGF-II gene from male chimaeras. In contrast, neonates that received the disrupted allele from their mother were normal sized. The difference in phenotype resulted because the paternal allele is expressed in most embryonic and fetal tissues, whereas the maternal allele supplements the paternal allele in the choroid plexus and leptomeninges only (DeChiara et al, 1990, 1991).

Interestingly the opposite is true for a receptor which binds IGF-II. Independently Bartlow et al (1991) discovered that the IGF type 2-receptor is predominantly expressed from the maternally derived chromosome. This would explain why mice develop normally if the paternal allele is missing but die during early devlopment if the maternal allele is missing. The evolutionary significance of parental origin-specific transcriptional regulation has been proposed in a recent review by Moore and Haig (1991).

Structure-Function Relationships

Insulin-like growth factor II is a single-chain polypeptide, consisting of 67 amino acid

residues with a predicted molecular weight of 7469 Da. The molecular weight, by mass spectrometry, was found to be 7440 +/- 4 Da. This difference in weight maybe a consequence of the acid extraction. The isoelectric point was found to be 6.7 whereas the 70 amino acid residue IGF-I peptide has a value of 8.8. This difference in isoelectric points was used to separate the two peptides during their original characterization (Van den Brande et al, 1990).

Comparison of the primary sequences of the IGFs, deduced either from the isolated peptide or from cDNA sequences, allowed recognition of domains similar to that of proinsulin: a B-chain-like N-terminal section (B-region in the IGFs), a short connecting peptide (C-region), and an A-chain-like section (A-region). The IGFs also have a carboxy-terminal extension (D-region), not present in proinsulin. The human preproIGF-II cDNA and protein sequences are depicted in Figure 2. The IGFs also share extensive homology with insulin at the amino acid level. All residues conserved in insulin between species are also conserved in the IGFs except for the last residue in the A-chain or A-region (residue 21) which is asparagine in insulin and alanine in the IGFs (Honegger, 1985). The conserved glycine and cysteine residues help in predicting the tertiary structure of the IGFs. The three dimensional structure of the IGFs have not yet been determined by x-ray crystallography or nuclear magnetic resonance, but computer-assisted molecular modelling has predicted a structure analogous to insulin (Humbel, 1984). Only in the past few years has the predicted disulfide bridges been verified for IGF-I, by using elaborate degradation and mass spectrometry techniques on recombinant IGF-I (Raschdorf et al, 1988), and for IGF-II using fast-atom-bombardment mass spectrometry (Smith et al, 1989). The most obvious differences from proinsulin are in the C-domain.

Along with the "classical" 67 amino acid IGF-II peptide, variant forms have been identified at both the protein and gene levels. A 10 kDa form isolated from human serum contains a tripeptide (Cys-Gly-Asp) substitution at Ser-33 as well as a carboxy-terminal extension E peptide of 21 amino acid residues (Zumstein et al, 1985). The tripeptide insert maybe a consequence of allelic variation since no splicing site at that position has

Fig. 2 ⇒

Figure 2. Sequence of human preproIGF-II cDNA and protein.

The amino acid sequence of preproIGF-II is numbered by designating the first amino acid of IGF-II as 1. Amino acids preceding the beginning of IGF-II comprise the signal peptide (given negative numbers) and are numbered sequentially from the Met residue, encoded by the ATG codon, at position -24. The region corresponding to IGF-II is boxed (residues 1-67). The B-domain of IGF-II comprises residues 1-32, the C-domain residues 33-40, the A-domain residues 41-61, the D-domain residues 62-67, and the carboxy-terminal E-domain residues 68-156.

-24 -20 -10

Met Gly Ile Pro Met Gly Lys Ser Met Leu Val Leu Leu Thr Phe

AUG GGA AUC CCA AUG GGG AAG UCG AUG CUG GUG CUU CUC ACC UUC

1

Leu Ala Phe Ala Ser Cys Cys Ile Ala Ala Tyr Arg Pro Ser Glu

UUG GCC UUC GCC UCG UGC UGC AUU GCU GCU UAC CGC CCC AUU GAG

10 20

Thr Leu Cys Gly Gly Glu Leu Val Asp Thr Leu Gln Phe Val Cys

ACC CUG UGC GGC GGG GAG CUG GUG GAC ACC CUC CAG UUC GUC UGU

30

Gly Asp Arg Gly Phe Tyr Phe Ser Arg Pro Ala Ser Arg Val Ser

GGG GAC CGC GGC UUC UAC UUC AGC AGG CCC GCA AGC CGU GUG AGC

40 50

Arg Arg Ser Arg Gly Ile Val Glu Glu Cys Cys Phe Arg Ser Cys

CUU CGC AGC CGU GGC AUC GUU GAG GAG UGC UUU UUC CGC AGC UGU

60

Asp Leu Ala Leu Leu Glu Thr Tyr Cys Ala Thr Pro Ala Lys Ser

GAC CUG GCC CUC CUG GAG ACG UAC UGU GCU ACC CCC GCC AAG UCC

70 80

Glu Arg Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe

GAG AGG GAC GUG UCG ACC CCU CCG ACC GUG CUU CCG GAC AAC UUC

90

Pro Arg Tyr Pro Val Gly Lys Phe Phe Gln Tyr Asp Thr Trp Lys

CCC AGA UAC CCC UUG GGC AAG UUC UUC CAA UAU GAC ACC UGG AAG

100 110

Gln Ser Thr Gln Arg Leu Arg Arg Gly Leu Pro Ala Leu Leu Arg

CAG UCC ACC CAG CGC CUG CGC AGG GGC CUG CCU GCC CUC CUG CUU

120

Ala Arg Arg Gly His Val Leu Ala Lys Glu Leu Glu Ala Phe Arg

GCC CGC CGG GUU CAC GUG CUC GCC AAG GAG CUC GAG GCG UUCAGG

130 140

Glu Ala Lys Arg Hls Arg Pro Leu Ile Ala Leu Pro Thr Gln Asp

GAG GCC AAA CGU CAC CUU CCC CUG AUU GCU CUA CCC ACC CAA GAC

150 156

Pro Ala His Gly Gly Ala Pro Pro Glu Met Ala Ser Asn Arg Lys

CCC GCC CAC GGG GGC GCC CCC CCA GAG AUG GCC AGC AAU CGG AAG

Stop
UGA

been identified. A variant with a tetrapeptide substitution at Ser-29, described previously, results from alternative RNA splicing at an intron-exon hinge region (Jansen et al, 1985). A 15 kDa form from human serum (Gowan et al, 1987), and several higher-molecular-mass forms have been found in human brain (Haselbacher et al, 1985). Most likely they represent partially processed forms of pro-IGF-II containing part of the E-domain. Another variant where Ser-29 is replaced by cysteine may be due to a single nucleotide substitution from A to T (Van den Brande et al, 1990).

The proposed three dimensional models of the IGFs along with known biological activities of normal and mutated peptides, provide a structural and physiological basis to explain the functional interactions of the IGFs and insulin with antibodies, IGF binding proteins and IGF receptors. Site directed mutagenesis of specific amino acids in IGF-I and IGF-II have been particularly useful in targetting of the IGF's exclusively to one of their two receptors. The main conclusions derived from these studies are as follows: (a) The region toward the end of the B-region was suggested to bind to the IGF-I receptor since a common area must be shared by insulin, IGF-I and IGF-II. The aromatic residues at positions 23-25 are definitely involved (Cascieri et al, 1988). (b) The binding to the type 2 receptor must involve a region not shared by insulin. Part of the A-region (at least positions 49-51) seems to be involved (Cascieri et al, 1989a). (c) The N-terminal part of IGF-I is necessary for the binding to IGF binding proteins (Ballard et al, 1987; Bagley et al, 1989; Cascieri et al, 1989b).

Insulin-like Growth Factor Receptors

Insulin and the IGF's are structural homologs which elicit, both long term effects on cell proliferation and short-term metabolic effects such as glucose transport, but with differing potencies. The response to hormones in the extracellular milieu by target cells is dependent on the presence of cell receptors as well as postreceptor mechanisms. Early competitive-binding studies indicated multiple IGF receptors that may have some heterogeneity among them (Hintz et al, 1972; Marshal et al, 1974; Megyesi et al, 1974;

Zapf et al, 1978; Rechler et al, 1980). Later, affinity crosslinking experiments have provided a physical basis for two distinct transmembrane high-affinity IGF receptors with different structural features and binding properties (Kasuga et al, 1981; Massague and Czech, 1982).

Type 1 IGF Receptor

Similar to the insulin receptor, the type I IGF receptor is a glycosylated heterotetramer, which consists of two extracellular α-subunits, containing the ligand binding sites, and two transmembrane β-subunits, containing the intracellular tyrosine kinase domain, linked by disulfide bonds (Kasuga et al, 1981; Chernausek et al, 1981; Massague and Czech, 1982). The alpha and beta subunits of the type I IGF receptor (or IGF-I receptor) are synthesized as a 1367 amino acid residue, single chain polypeptide precursor of 152 kDa when unglycosylated (NH_2-α-subunit-β-subunit-COOH). Its 30 residue signal peptide is removed during translocation into the endoplasmic reticulum. The remaining protein is then glycosylated, dimerized, oligosaccharide modified, linked by intrachain and interchain disulfide bonds, and finally, proteolytically processed to yield the mature β-α-α-β receptor complex (Jacobs et al, 1983; Duronio et al, 1986, 1988). The human IGF-I receptor gene has been mapped to the long arm of chromosome 15q25-26 (Ullrich et al, 1986). Its primary sequence was determined from placenta cDNA and found to have a high degree of amino acid homology with the insulin receptor, which was most pronounced in the tyrosine kinase domain (84% identity). This receptor preferentially binds IGF-I, IGF-II to a lesser degree, and binds weakly to insulin even at high concentrations. The binding affinities for the three ligands to the IGF-I receptor were found to be: IGF-I, K_d 1.5 nM; IGF-II, K_d ~3nM; insulin, K_d ~100nM (Steele-Perkins et al, 1988). Some discrepancies in the literature on receptor affinities may be due to two distinct binding sites, described using purified placental type I receptor. One site has high affinity for IGF-I which can be blocked by αIR3, an antibody against the IGF-I receptor, and the other site preferentially binds IGF-II which is not blocked by αIR3 (Casella et al, 1986).

In the precursor receptor, a tetrabasic cleavage sequence from amino acid residues 707 to 710, Arg-Lys-Arg-Arg, separates the α-subunit from the following 656 residues of the β-subunit. The α-subunit contains a cysteine-rich region (24 cysteines), between residues 148 and 302, also found in the insulin and EGF receptors. Although only a moderate overall homology is observed between the insulin receptor and IGF-I receptor in this cysteine-rich region, the placement of the cysteines is almost perfectly conserved. In the β-subunit, the 24 hydrophobic amino acid sequence beginning at residue 906 is followed by several basic residues; a characteristic of cytoplasmic anchors. Forty-three residues following the membrane spanning domain begins the highly conserved tyrosine kinase domain (amino acids 973-1229), which contains a Lys(1003) and a Gly-XXX-Gly-XXX-XXX-Gly motif that comprises the conserved, ATP-binding regions of all protein tyrosine kinases. A YETDYY sequence at position 1129 corresponds to the major site of autophosphorylation in the insulin receptor. In both insulin and IGF-I receptors, between the transmembrane domain and the tyrosine kinase domain, is an NPXY motif found to be necessary for receptor-mediated internalization of the LDL receptor (Ullrich et al, 1986; Chen et al, 1990).

Intrinsic tyrosine kinase activity is a common property among many growth factor receptors, and is thought to be involved in the signal pathway by which growth is stimulated by many growth factors and oncogene products. The binding of the IGFs to the extracellular α subunit region of the IGF-I receptor stimulates tyrosine autophosphorylation of the intracellular β subunits (Jacobs et al, 1983; Van Wyk, 1985). It is speculated that receptor cross-phosphorylation can occur between identical β subunits in the heterotetramer, as well as in hybrid receptors between β subunits of the insulin and the type I IGF receptor (Beguinot et al, 1988). Recently, hybrid receptors made up of one α-β insulin receptor heterodimer disulfide bonded to one α-β IGF-I receptor heterodimer have been proposed to explain the overlapping yet distinct physiological functions of insulin and IGF-I (Soos et al, 1990; Myers et al, 1993). Perhaps cross-phosphorylation also occurs between β subunits of adjacent receptors which may explain the amplified signal transmission observed during receptor aggregation (Ikari et al, 1988). By analogy

with the insulin receptor, tyrosines 1131, 1135, and 1136 appear to be the main sites of autophosphorylation in the IGF-I receptor (Moxham et al, 1989). Autophosphorylation results in the activation of the tyrosine kinase domain. Preincubation of the receptor with ATP has been shown to cause time-dependent activation kinase activation which was reversed by akaline phosphatase (Sasaki et al, 1985; Yu et al, 1986), indicating that tyrosine phosphorylation is essential for tyrosine kinase activity.

It is generally agreed that the receptor tyrosine kinase function of the IGF-I receptor is required to generate a cellular signal, however, there are few reports offering direct experimental evidence. For example, Izumi et al (1988) used an antibody specifically directed against the tyrosine kinase domain of the IGF-I receptor to inhibit IGF-I action. While these reports suggest that tyrosine kinase is directly involved in generating a cellular response, it is possible the antibody could also interfere with other necessary receptor functions, thereby inhibiting IGF-I. In contrast, a monoclonal antibody against the IGF-I receptor which prevents ligand binding, mimicked the effects of IGF-I to stimulate thymidine incorporation and glycogen synthesis in IGF-I receptor expressing CHO cells, without autophosphorylation or phosphorylation of cellular substrates (Steele-Perkins, 1988). Perhaps a biological response may also be produced by a secondary messenger mechanism totally unrelated to the normal tyrosine kinase cascade of protein phosphorylations.

Recently, tyrosine kinase activity in response to insulin or IGF-I has been shown to phosphorylate insulin receptor substrate 1 (IRS-1), a component of pp185 which functions as a docking molecule to facilitate phosphorylation of the regulatory subunit (p85) of the heterodimer, phosphatidylinositol 3-kinase (PI 3-kinase; Sun et al, 1991). The insulin and IGF-I receptors both generate a cytoplasmic signal pathway involving IRS-1-PtdIns-3 kinase (Endemann et al, 1990; Ruderman et al, 1990; Myers et al, 1993). Another major, endogenous substrates for the tyrosine kinase of the insulin and IGF-I receptors is pp240, however, its physiological function is not known (Kadowaki et al, 1987; Shemer et al, 1987). Map2 kinase, a serine/threonine-specific protein kinase important in regulating the

cell cycle (Boulton et al, 1990) is also activated when cells are treated with IGF-I (Hoshi et al, 1988). Map2 kinase is one of the very few serine/ threonine kineses with a phosphotyrosine. Other substrates for tyrosine kinases which may be activated by the IGF-I receptor include GTP activating protein (GAP), the GTPase effector of *ras*; *raf*, a serine/threonine proto-oncogene; and phospholipase C-γ.

Type 2 IGF Receptor

The type 2 IGF or Man-6-P(MPR)/IGF-II receptor is a single chain polypeptide of molecular weight ~260 kDa which is structurally quite different from that of the receptors for insulin and IGF-I. The primary amino acid sequence of the human IGF type 2 receptor was deduced from a cDNA clone isolated from the human hepatoma cell line HepG2 (Morgan et al, 1987; MacDonald et al, 1988). Very little homology was found with either the IGF-I or insulin receptor sequences. The primary sequence indicated that the M6P/IGF-II receptor consisted of a 44 residue amino-terminal signal sequence, a 2269 residue extracellular domain, a single 23 residue transmembrane spanning region, and a small 163 residue carboxyl-terminal cytoplasmic domain. The extracellular domain comprises 93 per cent of the total protein and is composed of 15 contiguous repeats of about 147 residues. These repeat units exhibit only 16 to 38 per cent sequence similarity; however, they have a clear pattern in the arrangement of 8 cysteine residues and hydrophobic regions, indicating that they have similar disulfide bonding within the repeats. Repeat # 13 contains a 43 residue insertion that is similar to the type II repeat of fibronectin.

The large extracellular domain of the M6P/IGF-II receptor contains numerous potential sites of N-linked glycosylations. In the presence of an N-linked glycosylation inhibitor, tunicamycin, a smaller 232 kDa receptor species accumulated which was unable to bind IGF-II (August et al, 1983; MacDonald and Czech, 1985). This suggests that, not only is the mature 260 kDa receptor glycosylated, but this glycosylation is necessary for IGF-II binding activity. At the present time it is not known which region of the extracellular

domain is involved in binding IGF-II. The IGF-II receptor gene has been mapped to the long arm of chromosome 6 (Laureys et al, 1988).

Another membrane-associated glycoprotein receptor which has similar binding specificities for phosphorylated oligonucleotides (M6P) was identified to have a molecular weight of 46 kDa and requires divalent cations for optimal ligand binding (Hoflack and Kornfeld, 1985). This receptor does not bind IGF-II and is refered to as the cation-dependent (CD) M6P receptor. Comparison of the amino acid sequence, deduced from the cloned cDNA, revealed 99.4 per cent identity with the human cation-independent (CI) M6P receptor, suggesting that the two receptors are the same protein (Oshima et al, 1988). Thus the much larger M6P/IGF-II receptor is interchangably called the cation-independent (CI) M6P receptor. There was an 80 per cent identity between human IGF-II receptor and the bovine CIM6P receptor (Lobel et al, 1987). A similar high degree of homology was observed with the rat placental IGF-II receptor sequence (MacDonald et al, 1988), further confirming that the IGF-II receptor was also the M6P binding receptor. The variation in there sequences may be attributable to evolutionary divergence.

Purified M6P/IGF-II receptor was initially shown to bind lysosomal enzymes via the monoester form of M6P residues in the carbohydrate portion of these glycoproteins, and also to M6P monomers themselves. Surprisingly only two of the 15 extracellular repeats are utilized for binding to M6P containing ligands (Tong et al, 1989). IGF-II binds at one site per receptor monomer (Tong et al, 1988). Binding of IGF-I is three orders of magnitude lower than the binding affinity of IGF-II to the IGF-II receptor. β-galactosidase (Kiess et al, 1990), β-glucuronidase (Nolan et al, 1990), and procathepsin-D (Mathieu et al, 1990) have been reported to inhibit the binding of ^{125}I-IGF-II to the M6P/IGF-II receptor. In contrast, mannose-6-P has been reported to actually increase the binding of IGF-II two fold to the IGF-II receptor (Roth et al, 1987; MacDonald et al, 1988). Presumably, incubation with M6P monomers strips off lysosomal enzymes from the receptor, resulting in an increase in affinity of the receptor for IGF-II (Roth et al, 1987; Polychronakoset et al, 1988). A physiological significance has yet to be attributed

to this synergistic relationship between IGF-II and M6P. A member of the TGF-β family has also been shown to be able to bind to the IGF-II receptor through M6P residues found on the biologically inactive precursor for TGF-β_1 (Purchio et al, 1988; Kovacina et al, 1989).

The IGF-II receptor shuttles between the plasma membrane, the trans-Golgi network, and the prelysosomal compartment. Its main function is to bind and physically separate lysosomal enzymes in the trans-Golgi network, from proteins destined for secretion, and targets them to the prelysosomal acidified compartments where dissociation of the ligand occurs (Pfeffer, 1988; Dahms et al, 1989). The receptors are then recycled to the trans-Golgi and the enzymes are delivered to lysosomes by membrane fusion (Duncan and Kornfeld, 1988). Lobel et al (1989) demonstrated, using IGF-II receptor mutants transfected into mouse L cells, that the cytoplasmic domain of the receptor contains structural features important for intracellular sorting and endocytosis. This mechanism for intracellular lysosomal enzyme targetting is refered to as the Biosynthetic Pathway. The majority of the IGF-II receptors are located intracellularly, but a small portion (~10 per cent) of the total number of cellular receptors are found on the cell surface. These receptors on the cell surface bind to extracellular lysosomal enzymes, which are typically secreted by cells at low levels (von Figura and Hasilik, 1986; Kornfeld, 1987). The ligand bound receptors internalize and release these enzymes in the endosomal compartment where they are then directed to the lysosomes. This M6P/IGF-II receptor mediated internalization of extracellular lysosomes and delivery to the lysosomes occurs via the Endocytic Pathway. The M6P/IGF-II receptor also mediates IGF-II internalization and degradation in the lysosomal compartments (Oka et al, 1985; Kiess et al, 1987).

Several studies have implicated the IGF-II receptor in the initiation of various biological responses to IGF-II. Most reports observed biological effects in response to IGF-II that were not duplicated by IGF-I. Protein phosphorylation, intracellular alkalization, and the generation of inositol triphosphate/diacylglycerol occured in response to IGF-II in both proximal tubule preparations and membranes from canine kidney (Hammerman and

Gavin, 1984; Mellas et al, 1986; Rogers and Hammerman, 1988). Similar effects were observed only with much higher concentrations of IGF-I. Only IGF-II but not IGF-I was able to stimulate DNA synthesis and growth of K562 cells, a subclone of the human erythroleukemia cell line (Tally et al, 1987). ^{3}H-thymidine incorporation in undifferentiated mouse embryonic limb buds, and glucose uptake, glycogen synthesis, and glycogen synthetase activity in both undifferentiated and differentiated limb buds were stimulated by IGF-II, but not IGF-I, *in vitro* (Bhaumick and Bala, 1988). IGF-II has also been observed to stimulate motility of Rhabdomyosarcoma cells via the IGF-II receptor (El Badry et al, 1990; Minniti et al, 1992).

Fewer reports used antibodies in order to verify that signaling was specific for the M6P/IGF-II receptor. Receptor immunoglobulins mimicked the effects of IGF-II by stimulating glycogen synthesis in a human hepatoma cell line (Hep-G2) (Hari et al, 1987), as well as DNA synthesis and Ca^{++} influx in Balb/c 3T3 cells that were primed with PDGF and EGF before the addition of IGF-II (Nishimoto et al, 1987a, 1987b; Matsunaga et al, 1988; Kojima et al, 1988). Further investigation of 3T3 fibroblasts determined that residues 2410 to 2423 of the receptors cytoplasmic domain can couple with G proteins to activate calcium channels. The IGF bound receptor activated $Gi\alpha_2$ and caused it to bind $GTP\gamma_S$, which in its unbound state increases the dissociation constant of IGF-II binding to the receptor, but when bound to $Gi\alpha_2$ allows a signal to be relayed. The phospholipase C mediated production of inositol triphosphate by the IGF-II receptor in canine kidney was inhibited by $GDP_{\beta S}$, while $GTP\gamma_S$ had no effect (Nishimoto et al, 1989; Okamoto et al, 1990; Murayama et al, 1990). These observations are also consistent with the premise that G proteins are coupled with a signal transmission by the IGF type 2 receptor.

Other studies, however, have shown that the effects of IGF-II are mediated by binding to the IGF type 1 or insulin receptors and not to the IGF-II receptor. Antibodies against the type 2 receptor which inhibit IGF-II binding does not effect IGF-II-stimulated DNA synthesis in H35 hepatoma cells (Mottola and Czech, 1984). In addition, insulin is 1000

times more potent than IGF-II in stimulating DNA synthesis, indicating that IGF-II was binding to the insulin receptor and not stimulating signal transduction through the IGF-II receptor (Massague et al, 1982; Koontz, 1984). Similar observations were made in fat cells (Mottola and Czech, 1984).

Insulin-like Growth Factor Binding Proteins

The regulation of hormone and growth factor action is an area of active investigation. One variable that provides control of cellular responsiveness to these factors at the extracellular level is the presence of high-affinity, soluble binding proteins. These large protein moieties may limit efflux of growth factors from the vascular space, limit access to receptors on cell surfaces, or directly target the growth factor to surface sites of action.

Many hormones and growth factors have been observed to be associated with binding proteins. Corticotropin-releasing hormone (Suda et al, 1988), growth hormone (Ymer and Herington, 1985), prolactin (Ymer et al, 1987), activin (Nakamura et al, 1990), epidermal growth factor (Drinkwater et al, 1987; Isackson et al, 1987; Pesonen et al, 1989), fibroblast growth factor (Dennis et al, 1989), platelet-derived growth factor (Huang et al, 1983; Raines et al, 1984), transforming growth factor β (Danielpour and Sporn, 1990), nerve growth factor (Greene and Shooter, 1980), and plasminogen activator inhibitor 1 (Declerck et al, 1988; Wiman et al, 1988) are among the many hormonal factors that seem to be inactivated by the formation of complexes with their binding proteins. Other binding proteins, such as those which bind bilirubin (Diaz-Gill et al, 1987) and the glycoprotein hormones (Pierce and Parsons, 1981), potentiate biological action. With the discovery of many more binding proteins for growth factors and hormones than generally appreciated, it is becoming clear that the presence of binding proteins may be a common mechanism by which cellular responsiveness to hormonal factors is regulated. The insulin-like growth factors are an important family of metabolic and mitogenic factors nearly always detected in association with high-affinity binding proteins (IGFBPs), which have been shown to function as modulators of IGF action (reviewed in Rechler, 1993;

Clemmons et al, 1993).

The complete primary structures of six forms of IGFBPs have now been determined from rat and human tissues and extracellular fluids, and are designated IGFBP-1 through 6 (Lee et al, 1988; Wood et al, 1988; Binkert et al, 1989; Shimasaki et al, 1990, 1991a, 1991b). These proteins have several common structural features and amino acid sequences of all six IGFBPs are greater than 70% conserved between rat and human species indicating that they are well conserved evolutionarily (Wood et al, 1988; Shimasaki et al, 1991c; Albiston and Herington, 1990; Murphy et al, 1990). IGFBPs are thought to inhibit IGF activity by the formation of IGF-IGFBP complexes that prevent binding of IGFs to their receptors. In certain circumstances, however, some IGFBPs may potentiate IGF action through mechanisms which are not completely understood. Other important biological properties of the IGFBPs include the ability to increase the circulating half-life of the IGFs and regulate their distribution (Davis et al, 1989; Guler et al, 1989; Hodgkinson et al, 1989). IGFBPs display tissue specific expression and the abundance of the different IGFBPs is regulated by different stimuli such as growth hormone or metabolic status. IGF-IGFBP-3 complexes bind to an acid-labile subunit, forming a large complex that is unable to leave the circulation, whereas the other IGFBPs can cross into tissues from the vascular space. IGFBP-6 and IGFBP-2 bind IGF-II with greater affinity than IGF-I (Roghani et al, 1989; Shimasaki and Ling, 1991) whereas the remaining binding proteins bind IGF-I and II with equipotency.

Biological Actions of IGF-II

The insulin-like growth factors are anabolic hormones with widespread action during embryonic, fetal and postnatal development. They regulate the proliferation and differentiation of a multitude of cell types and are capable of exerting insulin-like metabolic actions, such as stimulation of glucose uptake, as well as glycogen and lipid synthesis in adipose tissue. The end physiological result is dependent on the responsiveness of the target cell. Similar to insulin, they are found in circulation, but are

much more abundant, and are also locally produced by numerous organs and tissues. The IGFs, therefore, have the potential to act via endocrine as well as autocrine and/or paracrine mechanisms.

In Serum

The IGFs were originally discovered as endocrine hormones, but no endocrine cells were attributed to their production and secretion into circulation. Early studies indicated that several growth hormone responsive organs may be the source of IGFs (Hall and Bozovic, 1969). The majority of endocrine IGFs have been proposed to be released into serum by the liver, which expresses IGF message at concentrations much higher than any other organ (Mathews et al, 1986). Bone, however, where IGF-II is present in significantly larger quantities than IGF-I, has also been suggested as a source of circulating IGF-II (Bautista et al, 1991).

The concentrations of IGF-I and II in serum are 10 to 100 fold-higher than those required *in vitro* to stimulate a biological response (Hall and Sara, 1983, 1984; Baxter and Martin, 1986; Baxter, 1988; Zapf and Froesch, 1986; Sara and Hall, 1990). Nowhere else in the body are concentrations of IGFs as high as in blood. In adult man, normal concentrations of IGF-I and II are about 200 and 700 ng/ml, respectively (Daughaday and Rotwein, 1989). IGF-I and II combined are about 750 μg/l or 100 nM, which is about 1000 times more than the concentration of insulin. Since the IGFs stimulate a decrease in blood glucose of approximately 5% the potency of that of insulin (Guler et al, 1987), this combined IGF concentration would be sufficient to provoke hypoglycemia unless the greater part of the IGFs were hindered from binding to the insulin/IGF type 1 receptor by IGFBPs (Baxter, 1988; Binoux and Hossenlopp, 1988).

The radioimmunoassay and radioreceptor assay are standard radioligand assay procedures to determine IGF-I and II measurements, respectively (Furlanetto and Marino, 1987; Daughaday, 1987). Since 95% of the IGFs in serum are associated with IGFBPs, epitopes

in the IGFs recognized by antibodies may be partially blocked by any of the IGFBPs. Therefore, the IGFBPs would interfere with the antigen-antibody reaction by competing with the antibody. As a result only a portion of the IGF content could be measured. Thus, complete dissociation and removal of the IGFs from their binding proteins is a prerequisite for quantitative analysis of the IGFs. The IGFs have also been found associated with IGFBPs in lymph (Binoux and Hossenlopp, 1988) and exocrine fluids, such as breast milk (Corps et al, 1988), amniotic fluid (Chochinov et al, 1977), and saliva (Costigan et al, 1988). Dissociation is easily achieved by acidification. Various techniques for separation, such as gel filtration (Zapf et al, 1981), C18 chromatography on SepPak cartridges (Daughaday et al, 1980; Davenport, 1988), centrifugation or automated procedures have been used (Bang et al, 1990). The discrepancies in the reported concentrations of IGFs are often due to different assay techniques and the lack of a common international standard. These difficulties, however, were resolved with the advent of commercially available recombinant IGF-I and II.

The high concentration of IGF-II in adult and, to a lesser extent, fetal serum of most vertebrate (with the exception of adult rodent) strongly suggests an endocrine role. IGF-II is detectable in human fetal plasma from at least fifteen weeks gestation, and maybe synthesized during early embryogenesis (Ashton et al, 1985). The quantity of IGF-II in the fetus is much more abundant than IGF-I. The circulating ratio of IGF-II : IGF-I is 4.4, which agrees well with the 4.8 ratio of IGF-II : IGF-I peptides extracted from human fetal liver, reinforcing that circulating IGFs are mostly synthesized within liver (Hill, 1990). From thirty-two weeks until birth, concentrations of both IGF-I and II increased by as much as two-fold, but still remains lower than in normal adult (Gluckman and Brinsmead, 1976; Ashton and Vesey, 1978; Zapf et al, 1981; Bennett et al, 1983). IGFs do not cross the placenta, and, therefore, those found in fetal blood are considered to be synthesized by fetal tissues (Underwood and D'Ercole, 1984). After birth, amounts of IGF-II rise to adult levels during the first year of life, and remain unchanged throughout life. The IGF-II levels in serum are four-fold higher than those of IGF-I in adult human (Zapf et al, 1981; Enberg and Hall, 1984), and in contrast to IGF-I the expression of IGF-

II is not growth hormone regulated (Daughaday et al, 1959; Almqvist et al, 1961; Copeland et al, 1980).

The biological role of growth hormone regulated IGF-I in postnatal growth is well established (Humbel, 1984; Van Wyk, 1984; Zapf et al, 1984). The human pubertal growth spurt results from increased secretion of growth hormone, which causes a 2 to 3 fold rise in serum IGF-I (Luna et al, 1983; Silbergeld et al, 1986). The IGF-I then acts on the proliferating chondrocytes of the long-bone growth-plates (Isakson, 1987). No significant changes in IGF-II concentrations occur during puberty (Zapf et al, 1981). The biological role of this far more abundant IGF-II peptide during postnatal life remains elusive.

In other animal species where fetal IGF levels have been investigated, IGF-II levels are greater in late fetal development than in the adult. In the rat, serum IGF-II concentrations in late gestation are 20 to 100 fold higher than in maternal serum (Moses et al, 1980; Daughaday et al, 1982) and falls dramatically by the time of birth. In fetal lambs, serum IGF-II is two-fold greater than maternal IGF-II (Gluckman and Butler, 1983), which declines abruptly to low levels before birth, and persist into the neonatal period. A similar pattern of expression to that of rat and lamb is observed in fetal quinea-pig (Daughaday et al, 1986). In the rat, circulating IGF-II nearly disappears from serum by the time of weaning, and is subsequently considered mainly as a fetal and neonatal growth factor. The rat IGF-II gene lacks the first promoter present in the human IGF-II gene, which gives rise to a liver-specific mRNA and serum protein in adult human (De Pagter-Holthuizen et al, 1988; Sussenbach, 1989).

Advances in DNA technology, which made available large quantities of purified recombinant IGF-I and II, have allowed for more definite studies on the effects of the IGFs. Schoenle et al (1982) were the first to show that subcutaneous infusion of IGF-I in the rat increased body weight, the width of the tibial epiphyses, and thymidine incorporation into costal cartilage. In Snell dwarf mice, which have low levels of growth

hormone and IGF-I, both IGF-I and II stimulated growth (Van Buul-Offers et al, 1986, 1988). The growth-promoting effect of IGF-II was found to be much less potent than IGF-I in adult rats (Schoenle et al, 1982, 1985). Postpubertal mice and rats, however, may not be a suitable model for evaluating the endocrine role of IGF-II since it disappears from rodent circulation at the time of weaning and is predominately present during fetal development in these species. Using a fetal model, Liu et al (1989) assessed the growth promoting effect of IGF-II in early rat development and found that renal arterial infusion of IGF-II significantly enhanced the growth of fetal rat paws in comparison to IGF-I. The role of endocrine IGF-II is still not clear, but is considered to integrate the whole-body physiology in the maintenance of balanced growth, anabolism and perhaps nutritional homeostasis.

Autocrine/Paracrine Actions

Somatomedins were initially considered to be derived from the liver, but their expression was also observed by several target organs and tissues for growth hormone (Hall and Bozovic, 1969). Since then, however, their expression and local production have been demonstrated in numerous organs and tissues of many animal species, which has challenged the original somatomedin hypothesis (D'Ercole et al, 1980; Mathews et al, 1986; Roberts et al, 1987; Hynes et al, 1987). This wide-spread expression is difficult to assimilate into a classical endocrine mode of action. The IGFs appear to function in a co-ordinated manner between serum and locally produced factors. A substantial part of the growth hormone stimulated growth promotion of long bones, for example, is mediated by paracrine production of IGF-I (Isakson, 1987). It is now generally accepted that the IGFs act as endocrine hormones via the blood and as autocrine and paracrine growth factors locally.

The IGFs have functional similarity with other growth factors which have both autocrine and paracrine modes of action, such as the transforming growth factors and the platelet derived growth factors. Like these other factors, the IGFs are pleiotropic modulators of

multiple aspects of cell physiology. Their biological activities include proliferation and nutritional homeostasis via mitogenic and anabolic actions, as well as induction and maintenance of differentiation. The IGFs, however, have a much wider anatomical and developmental range of gene expression and peptide localization than any other known peptide growth factor.

Brice et al (1989) used *in situ* hybridization histochemistry to show that blastocysts, produced by *in vitro* fertilization, did not express IGF-II mRNA before implantation indicating a lack of dependence of the early embryo on endogenous IGFs for growth and differentiation. First expression of IGF-II mRNA appears to be between 5 and 18 days post-fertilization. Studies with embryonal carcinoma cell lines suggest that the IGF-II gene is only expressed after their differentiation into mesodermal and endodermal elements. Following differentiation the cells become sensitive to exogenous IGF-I and II, and have type I receptors (Nagarajan et al, 1985; Heath and Shi, 1986). The pattern of IGF-II expression in different tissues is age related. In the kidney, IGF-II mRNA was found mainly in the cells of metanephric blastema and decreases as the blastema differentiates, whereas in the placenta, the expression was in the cytotrophoblast layer and it appeared to increase with differentiation. This expression in a temporally regulated fashion may be due to the appearance of a differentiated cell type, resulting in the disappearance of IGF-II mRNA. Similar observations were made in the fetal rat (Beck et al, 1987). This pattern of early expression differs from that of older fetuses.

From 12 weeks gestation in humans, both IGF-I and II mRNAs are detectable in almost every tissue (Han et al, 1987a, 1988; Gray et al, 1987). Before 12 weeks it is difficult to dissect out tissues and organs separately, therefore it is likely that IGF-I and II may be synthesized in human tissues during early embryogenesis. Significantly higher levels of IGF-II mRNA were detected suggesting that in early fetal tissues, similar to serum, IGF-II is synthesized in greater quantities than IGF-I. Immunoreactive IGF-I and II that could be extracted from a variety of 14 to 16 weeks fetal tissues had a mean IGF-II : IGF-I ratio of only 3.3 (Han et al, 1988). An unusual feature of human fetal tissues, serum, and

cultured medium conditioned by human fetal cell types is that upto half of the immunoreactive IGF-II is of the "big form", that is between 8 and 15 kDa, in addition to the mature 7.5 kDa peptide (Hill, 1990). These large molecular weight IGF-II species may represent the translated products of different mRNA species or incompletely processed peptides from a single transcript.

The levels of IGF-II transcript in human fetal tissues have been studied by several groups (Gray et al, 1987; Han et al, 1988; de Pagter-Holthuizen et al, 1987, 1988, 1989; Schofield and Tate, 1987; Scott et al, 1985; Shen et al, 1986). Generally, a high concentration is found in liver, adrenal gland, kidney, and skeletal muscle; intermediate levels in placenta, pancreas and heart; and low levels in lung, intestine, brain, spleen and thymus. Studies by *in situ* hybridization histochemistry localized IGF mRNA to fibroblasts, connective tissues or other cells of mesenchymal origin and not in the epithelial cells, where immunoreactive IGFs were localized (Han et al, 1987a, 1987b). Similar immunocytochemical studies in developing rodent (Beck et al, 1987; Stylianopoulou et al, 1988a) and chick embryo (Ralphs et al, 1990) are in general agreement with this cellular localization to mesoderm derivatives (primitive mesenchyme). The above immunoreactive studies, therefore, identify IGFs at their sites of action, bound to cell-surface receptors or BPs, and not at the cells of IGF synthesis; thereby, speaking strongly in favor of a paracrine mode of action of the tissue IGFs. Localization of IGFBP immunoreactivity to cellular sites identical to those of the peptides appear to confirm this hypothesis (Hill et al, 1988).

It would appear that if the IGFs are important regulators of tissue growth an association would be difficult to make by simply measuring peptide concentrations since growth factor activity is regulated by mechanisms beyond just transcription and translation. Perhaps the biological activity of the abundant tissue IGF levels through gestation are regulated by changes in the tissue expression of functionally different IGFBPs. In addition, the IGF biological activity occurs in a manner coordinated with different growth factors and growth regulators. There are synergestic interactions between the IGFs and

other peptide growth factors, such as fibroblast growth factor or epidermal growth factor, during DNA synthesis in fetal cell cultures (Kaplowitz et al, 1982). This is due to specific and differing roles of each growth factor at the G0/G1 boundary or during the G1 phase of the cell cycle described earlier (Van Wyk et al, 1981). It is likely that IGF-II is an impotant part of a repertoire of growth factors and growth regulators and thus is important but not exclusively essential. DeChiara et al (1990) used homologous recombination to introduce mutations at the IGF-II gene locus in mice. Heterozygous progeny showed a growth deficient phenotype of about 60% the body weight of their normal wild type littermates. These IGF-II deficient mice were otherwise normal and fertile indicating a physiological role of IGF-II in embryonic growth. The "knock-out" of IGF-I and/or the type 1 IGF receptor in mice by the same homologous recombination technique resulted in profound fetal growth retardation (Liu et al, 1993; Baker et al, 1993) which confirms a fundamental importance of the IGF system in development.

Growth alone may not be the only physiological function of IGF-II during gestation. The IGFs also influence the rate of cellular differentiation of fetal and neonatal cell types from muscle, cartilage, bone, brain, and adrenal gland. In many instances this influence on differentiation can functionally seperated from their actions on cell proliferation (Ewton and Florini, 1981; Burch et al, 1986; Froesch et al, 1976; Hill, 1979; Binoux et al, 1985; Han et al, 1987c; Recio-Pinto and Ishii, 1984; McMorris and Dubois-Dalq, 1988; Challis and Olson, 1988). In the developing chick embryo, the distribution of IGF peptides was associated more obviously with sites of tissue differentiation then with sites of rapid cell proliferation which agrees with previous studies using early fetal tissues (Ralphs et al, 1990).

Breast Cancer and other Malignancies

The ability of transformed or tumor-derived cell lines to proliferate *in vitro* in the absence of serum led investigators to postulate that such cells were capable of secreting self-sustaining factors (Engstrom and Zetterberg, 1983). From these early reports emerged the

concept that the reduced requirements for serum resulted from *de novo* synthesis of polypeptide growth factors by the tumor cells themselves. The growth factors then act by direct or indirect autocrine loops to stimulate the proliferation of the same cell or same cell type. Subsequent analysis identified that tumor cells had an abnormal capacity to produce autocrine growth factors (Sporn and Roberts, 1985b). In addition, the discovery that many transforming oncogenes are altered forms of growth factors, growth factor receptors, or secondary messenger components led to the realization that amplified or dysregulated growth factor control may play an important role in tumorigenesis (Doolittle et al, 1983; Waterfield et al, 1983; Downward et al, 1984; Dickson and Peters, 1987; Tiara et al, 1987). The acquisition of an altered growth phenotype which depends on these factors, may release an autocrine cell population from normal growth restraints and result in a selective growth advantage.

The first clear evidence that IGF-II could act locally in an autocrine or paracrine mode was provided by Dulak and Temin (1973) using Buffalo rat liver cells, and was subsequently verified by Marquardt et al (1981). The discovery that rodent fibroblasts of fetal origin (Adams et al, 1983) and the human neoplastic fibrosarcoma cells (DeLarco and Todaro, 1978) grown *in vitro* produce IGF-II and release them into the culture medium led workers to examine synthesis of IGFs in other developmental tumor cell lines. In a historical context, workers initially examined murine and human teratocarcinomas, and later rhabdomyosarcoma, Wilms' tumor and related embryonal neoplasms (Scott et al, 1985; Heath and Shi, 1986). This was followed by a much more extensive investigation of a wide range of tumor types, a number of which were found to have elvated levels of IGF-II mRNA (Gray et al, 1987; Haselbacher et al, 1987; Hoppener et al, 1988; Reeve et al, 1985; Sandberg et al, 1988; Scott et al, 1985; Su et al, 1989; Tricoli et al, 1986).

In many naturally occurring and experimental tumors the 'fetal' IGF-II promoters are activated, with the 6.0 and 2.2 kb transcripts of promoter P2 being the most abundant ones detected. Similar to these cells' fetal counterparts, these tumors: make significant

quantities of IGF-II mRNA (Scott et al, 1985; Schofield and Tate, 1987; Brice et al, 1989; Hirvanen et al, 1989; Wilkins et al, 1989); frequently lack the enzymatic machinery to completely process proIGF-II to the final 7.5 kDa peptide, resulting in predominantly big IGF-II being present (Daughaday et al, 1988; Haselbacher et al, 1987); and the most striking similarity is the rapid cellular proliferation. These properties are not observed in normal non-neoplastic cellular counterparts.

The temporal relationship that exists between the increase in IGF-II and tumor formation raises questions concerning the role of IGF-II in tumorigenesis. Consistent elevation of IGF-II transcripts correlate with: i) the depth of tumor invasion through the bowel wall in both liposarcomas and colon carcinomas (Tricoli et al, 1986); ii) the occurrence of leiomyosarcomas compared to their benign counterparts (Hoppener et al, 1988; Gloudemans et al 1990); and, iii) the occurrence of hepatic carcinogenesis of liver nodules in the adult rat (Norstedt et al, 1988). Similarly, there is a shift from promoter P3 to P2 usage in adult smooth muscle tumors as malignancy increases (Hoppener et al, 1988; Sussenbach et al, 1991). This shift in IGF-II gene expression during tumorigenesis is likely due to differential expression of genes encoding transcription factors or regulatory proteins binding to the 'fetal' promoters of the IGF-II gene, rather than direct mutation of the IGF-II promoter in the tumor (Sussenbach et al, 1991).

In the case of leukemias and some other carcinomas a cooperative paracrine relationship has been observed. The synthesis of PDGF by tumor cells may induce synthesis of IGFs by the surrounding stromal tissue as part of a paracrine interaction between the two cell types in a tumor (Clemmons, 1984; Pepe et al, 1987). These tumors often lack receptors for PDGF, but contain IGF receptors, and in this way set up an indirect autocrine loop to support each others growth. A primarily stromal origin of IGFs is supported by the fact that IGF overexpression has been reported in a number of sarcomas, which are of mesenchymal origin without a predominant epithelial component (Hoppener et al, 1988; Tricoli et al, 1986). An identical relationship is observed between surrounding stromal fibroblasts and malignant breast lesions (Cullen et al, 1991).

When breast tumor specimens were examined from patient biopsies, RNAse protection assay detected IGF-II mRNA in 24 of 26 cancer specimens. Four out of five human skin fibroblasts cells lines also expressed IGF-II message suggesting that the source of IGF-II mRNA in tumors is predominantly stromal rather than epithelial (Arteaga et al, 1989). In order to further address this, growth factor expression in primary cultures of stromal fibroblasts derived from benign and malignant breast lesions were examined to identify stromal growth factors which might be important in the overall growth regulation of these neoplasms (Cullen et al, 1991). All fibroblasts expressed PDGF A chain, TGF-β1, FGF and FGF-5 mRNAs, while none expressed the PDGF B chain or TGF-α mRNAs whether cultured fibroblast studied originated from a benign or a malignant lesion. Only the expression of the IGFs correlated with the type of cell examined. Seven of eight fibroblasts derived from benign lesions and only one of nine lines derived from malignant tumors expressed IGF-I, while the opposite was seen for IGF-II expression. One of nine benign-derived fibroblasts from benign lesions expressed IGF-II while most (5 of 9) fibroblasts derived from malignant tumors produced IGF-II transcript. This pattern of IGF expression in stromal cells was confirmed by Paik (1992) who performed *in situ* hybridization on paraffin-embedded sections of malignant and benign breast biopsy specimens.

Of the above growth factors, only the IGFs have the most clearly defined mitogenic effects on tumor epithelial growth. Fibroblast production of PDGF would presumably not affect tumor growth because breast tumor epithelial cells *in vitro* lack receptors for PDGF and do not respond to it in biological assays (Bronzert et al, 1987), whereas TGF-β1 acts as an inhibitor (Tucker et al, 1984; Roberts et al, 1985). FGF is likely to be more important in the stimulation of angiogenesis, which is necessary for tumor growth to continue, although it has been shown to also be mitogenic (Nonomura et al, 1990). Although breast tumor epithelial cells are unable to respond to PDGF, they produce both PDGF A and B chains, which have been shown to accelerate growth of stromal fibroblasts. PDGF also induce production of IGF-II threefold in a breast tumor fibroblast line *in vitro* (Cullen et al, 1991). IGF-II in turn stimulates greater epithelial production

of PDGF, resulting in a possible cooperative paracrine loop seen in other tumors described previously.

IGF-II mRNA is also detected in some normal breast fibroblasts (Paik, 1992), is abundant in skin fibroblasts (Singer et al, 1992), and the differences in IGF expression in fibroblasts surrounding neoplasms appear to remain even after being seperated from their associated epithelial cells (Cullen et al, 1991); thus, the expression of IGF-II in stromal fibroblasts from breast tumors is likely the result of the selection of and expansion of a pre-existing subpopulation of fibroblasts rather than a transformation event within those cells. These findings suggest that IGF-II may be an important growth promoter in malignant lesions predominantly via a paracrine mechanism.

The first step of IGF-II action is its binding to specific membrane receptors (Rosenfeld and Hintz, 1988). Although IGF-II preferentially binds to the type 2 IGF receptor, the majority of its mitogenic effect is blocked by αIR3, a monoclonal antibody directed against the type 1 IGF receptor (Osborne et al, 1989; Cullen et al, 1990). This antibody, however, had no effect at low IGF-II concentrations in MCF-7 cells (Mathieu et al, 1990), suggesting that the type 2 receptor mediates IGF-II effects at low concentrations in these cells (Cullen et al, 1990). Similarly α-IR3 inhibited most, but not all, of the IGF-II mitogenic in MDA-MB-231 ER negative cells (Osborne et al, 1989). In vivo, the type 2 IGF receptor also mediates some of the IGF-II growth action in T61 xenotransplants (Brunner et al, 1993). A mutant IGF-II peptide containing a leucine substitution for tyrosine at residue 27, which had reduced binding to the type 1 receptor by approximately 100 fold, while not affecting type 2 receptor binding, was expresed in Balb/c 3T3 fibroblasts. Thymidine incorporation also drastically reduced in these cells (Beukers et al, 1991). Thus the type 1 receptor predominantly mediates the growth effect of IGF-II but the type 2 IGF receptor may have physiological significance in some cells.

The expression of both type 1 and 2 IGF receptors were characterized in order to explore the biological role of these receptors in human breast cancer. Virtually all breast tumor

specimens and breast cancer epithelial cell lines examined expressed mRNA for both IGF receptors (Cullen et al, 1990; Peyrat et al, 1990). In addition to the typical insulin, IGF type 1 and 2 receptors, an atypical receptor that binds both insulin and IGF-I with high affinity has been detected in MCF-7 cells (Milazzo et al, 1992).

When the type 1 receptor was assayed in a large series of breast cancer biopsies, it was present in most cases (Pollak et al, 1987; Peyrat et al, 1988a; Pekonen et al, 1988; Foekens et al, 1989; Bonneterre et al, 1990). Lower levels of type 1 IGF receptor were found in benign breast disease, whereas little or no receptor was found in normal breasts or normal tissue adjacent to tumor tissue (Pekonen et al, 1988; Peyrat et al, 1988b, Bonneterre et al, 1992) indicating that the IGF family are important in breast cancer. Interestingly, a positive relationship was found between the type 1 IGF receptor and ER or progesterone receptor (PgR; Bonneterre et al, 1990). This correlation was stronger in post-menopausal women. Since the presence of ER and PgR is also associated with better prognosis, the development of IGF antagonists or compounds that control type 1 IGF expression, in conjunction with ER and PgR antagonists, could be useful in treatment of these tumors. Antibodies directed against the type 1 IGF receptor have inhibited breast cancer growth *in vitro* and *in vivo* (Rohlik et al, 1987; Arteaga et al, 1989; Arteaga and Osborne, 1989; Brunner et al, 1993).

A few reports of breast tumor epithelial cells expressing IGF-II mRNA *in vitro* and *in vivo* suggest that in some cases IGF-II may also be able to act as an autocrine growth promoter (Paik et al, 1992; and references below). Previously, we reported that IGF-II was a potent mitogen for human breast cancer (T47D) cells (Myal et al, 1984), which was confirmed by many investigators using other estrogen receptor (ER)-positive and ER-negative tumor epithelial cell lines (Karey and Sirbasku, 1988; Osborne et al, 1989; Cullen et al, 1990). Although very few cultured breast tumor epithelial cell lines express IGF-II mRNA, transcripts present in the ER-positive, T47D cell line and a MCF-7L subline, were also estrogen inducible (Yee et al, 1988; Osborne et al, 1989; Arteaga et al, 1989). Both Northern blot analysis and RNAse protection assay showed a 2 to 5-fold

increase in IGF-II mRNA after 8 hours of treatment with 1nM estradiol. The weak estrogen, phenol red, also increased IGF-II expression and was blocked by pretreating cells with tamoxifen (Arteaga et al, 1989; Osborne et al, 1989).

A human ER+ breast tumor xenograft (T61), established from a primary breast cancer, was successfully grown in athymic nude mice (Brunner et al, 1992, 1993). ER+ MCF-7 and ER- MDA-MB-231 xenografts were also established in parallel. All xenografts expressed IGF-II along with type 1 and 2 IGF receptors. In ER+ tumors, growth and IGF-II expression was regulated by estrogen. Estrogen increased IGF-II levels in MCF-7 cells and was required for tumor formation. The opposite was seen in the T61 xenograft. T61 tumor growth, IGF-II mRNA and protein expression was inhibited by treatment with estrogen and tamoxifen. This down regulation of IGF-II was specific for IGF-II since no changes in the expression of IGF-I, 36B4, TGF-α, or EGF receptor mRNAs were detected. While tamoxifen caused a stabilization in tumor size, estrogen induced tumor regression. Treatment with α-IR3 also resulted in inhibition of tumor growth, which recommenced when treatment stopped. Therefore estrogen stimulation of breast cancer cell proliferation *in vitro* and *in vivo* may be mediated by IGF-II production acting via an autocrine mechanism.

This nude mouse model provides important interactions between the tumor cells and the hosts fibroblasts, endothelial cells, and other mouse cells, as well as providing a functioning endocrine system which are not addressed in a purely *in vitro* system. It is difficult, however, to assess how far such a system is representative of the *in vivo* state.

Two independent investigators also found that stable MCF-7 clones transfected with expression vectors containing the coding sequence for preproIGF-II induced phenotypic alterations associated with malignant progression such as estrogen and anchorage independent growth (Daly et al, 1991; Cullen et al, 1992). Control cells infected with an identical vector containing irrelevant DNA sequence, did not express IGF-II mRNA or protein, and had no phenotypic differences from wild-type MCF-7 cells. Thus, IGF-II

stimulation of breast cancer epithelium, whether being paracrine or autocrine stimulation, results in a significant proliferation and/or transformation response. However, other investigators have observed IGF-II features not satisfied by a paracrine or autocrine mode of action.

The secretion of autocrine GFs such as TGF-α and IGF-II mediating estrogen action in human breast cancer cells was under question when the antibody blockade against epidermal GF (EGF) and/or IGF-I receptors, which inhibits ligand binding, failed to inhibit estrogen induced growth (Arteaga et al, 1988, 1989; Arteaga and Osborne, 1989; Arteaga, 1992). The α-IR_3 antibody, however, did inhibit breast cancer cell growth in serum (Arteaga and Osborne, 1989). In addition, a tyrosine kinase inhibitor (RG-13022), which passively enters the cell, did inhibit the estrogen induced growth of both T47D and MCF-7 cells (Reddy et al, 1992). These conflicting results could be explained by an interaction between IGF-II and/or TGF-α with their receptors intracellularlly resulting in transduction of a mitogenic signal without secretion into the extracellular fluid. This intracrine mode of action has been observed by other growth factors (Bejcek et al, 1989; Dunbar et al, 1989; Taylor et al, 1993; Sherman et al, 1993; Huang et al, 1994) and has been postulated as another possible mechanism by which IGF-II stimulates proliferation of human breast cancer cells *in vitro* (Reddy et al, 1992; Dubois et al, 1993) and *in vivo* (Arteaga, 1992; Yee, 1992).

The autocrine and paracrine stimulation of human breast cancer cells by IGF-II and/or other growth factors may be one reason for the failure of response to endocrine treatments (Anderson et al, 1989). Synthetic antagonists able to interfere with growth factor stimulated proliferation by blocking receptor binding or the receptor mediated signal transduction mechanism could therefore represent a promising new therapeutic strategy. Pharmacological administration of the monoclonal antibody, α-IR3, inhibited MDA-MB-231 tumor formation in athymic mice (Arteaga, 1992) and the growth of T61, a human breast tumor xenograft (Brunner et al, 1993). If IGF-II is able to act intracellularly, such cells would not be susceptible to treatment strategies using external antibodies.

C. Intracrine Action of Growth Factors

Polypeptide growth factors have emerged over the last decade as a group of trophic regulatory substances distinct from hormones. Their shared characteristics include the regulation of an array of cellular functions by receptor-mediated mechanisms but differ primarily in their cell of origin and method of transport. As noted earlier, endocrine hormones are produced by specialized cells in discrete glands and are transported to distinct target cells by means of the blood stream. Growth factors are produced from a wide variety of cell and tissue types and travel predominantly by diffusion to target cells situated in close proximity bearing receptors. Such trophic factors which regulate the growth of their surrounding cellular environment are called paracrine factors, while cells which produce the cell surface receptors for trophic factors they secrete may regulate their own growth and thus exhibit autocrine interactions.

In the classical model of an autocrine loop, an autocrine growth factor is secreted into the extracellular fluid before binding to its receptor on the same cell or neighbouring cells. Several distinct features have been observed that are consistent with this model. They include: the secretion of biologically active growth factor into the culture medium and additive stimulation of growth by exogenous growth factors; cell growth characteristics which were density-dependent; and the inability to proliferate in the presence of neutralizing antibodies specific for the growth factor (Duprez et al, 1985; Young and Griffin, 1986; Arima et al, 1986; Scala et al, 1987; Laker et al, 1987; Rambaldi et al, 1988). The secreted factor, into the extracellular fluid, may also act in a paracrine manner on other surrounding cell types and stimulate the secretion of additional factors resulting in the creation of more complex interactive loops.

Secretion has conventionally been considered to be a prerequisite of growth factor bioactivity, but evidence is emerging to support an alternate mechanism by which growth factors may act intracellularly, termed an intracrine mechanism of action. This intracrine

pathway is supported by observations that several secreted and transmembrane proteins comigrate in the same endoplasmic reticulum cisternae, Golgi apparatus, and secretory vesicles (Strous et al, 1983), theoretically allowing binding of a growth factor to its receptor in these compartments (Figure 3). In contrast to the classical autocrine characteristics, investigators have observed cell growth that is not influenced by the amount of growth factor secreted; cell proliferation is independent of cell density, even in the absence of exogenous factors; and neutralizing antibodies do not prevent continued growth (Lang et al, 1985; Brown et al, 1987; Fleming et al, 1989; Browder et al, 1989). These findings support a mechanism by which the growth factor remains within the cell of origin, binds to its receptor, and stimulates intracellular messengers to regulate cellular function. Growth factors acting in this way need not be secreted, nor do they require receptors located on the cell surface to mediate their functions, and could result in a selective growth advantage for these cells particularly if growth factor secretion were low or absent.

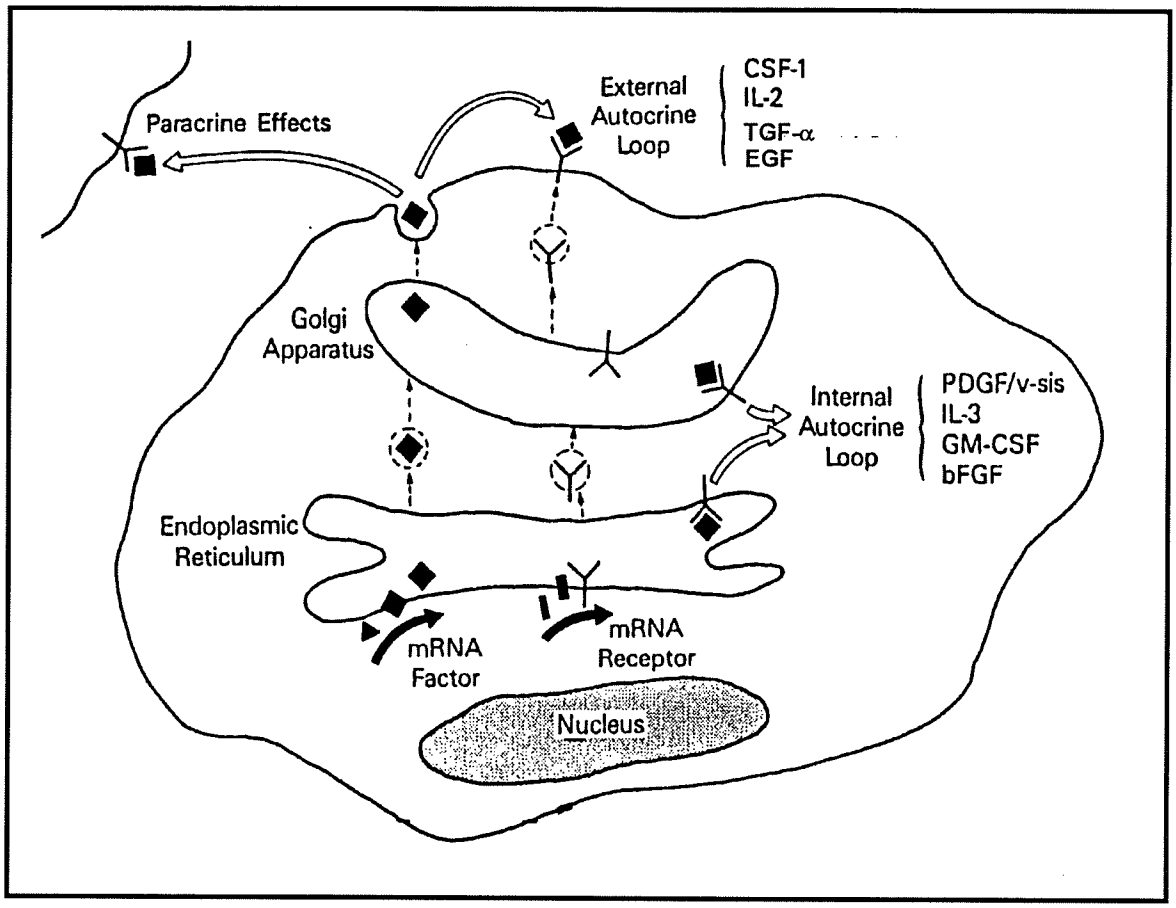
D. KDEL-Endoplasmic Reticulum Intracellular Retention Mechanism

Eukaryotic proteins destined for secretion or residence within membranes via the secretory pathway undergo co-translational translocation into the lumen of the endoplasmic reticulum (ER) in response to topogenic or signal sequences found on newly synthesized proteins (Blobel and Dobberstein, 1975; Lingappa et al, 1991; Simon and Blobel, 1991). The lumen of the ER is densely packed with soluble resident proteins (Weibel et al, 1969; Freedman, 1984) such as the immunoglobulin heavy chain-binding protein (BiP) which is involved in protein oligomerization (Haas and Wabl, 1983), and protein disulphide isomerase (PDI) which catalyzes thiol/disulfide interchange reactions and promotes protein disulfide formation, isomerization and reduction (Edman et al, 1985;

Fig. 3 ⟹

Figure 3. The cellular pathway followed by growth factors and growth factor receptors during their biosynthesis.

An illustration of the possible growth factor interactions with their receptor(s) in intracellular compartments which may result in intracrine growth stimulation. Also shown schematically is receptor binding at the cell surface of the growth factor producing cell as well as neighboring cells resulting in autocrine and paracrine effects, respectively.


Paracrine Effects
External
Autocrine
Loop
CSF-1
IL-2
TGF-α
EGF
Golgi
Apparatus
Internal
Autocrine
Loop
PDGF/v-sis
IL-3
GM-CSF
bFGF
Endoplasmic
Reticulum
mRNA
Factor
mRNA
Receptor
Nucleus

Gething and Sambrook, 1992). Many of the resident ER proteins are involved in protein assembly (Rothman, 1989). They bind nascent polypeptides that have crossed the ER membrane and catalyze proper assembly and folding. Vesicular transport of newly assembled soluble proteins to the Golgi apparatus seems to be non-specific and does not require a signal sequence (Palade, 1975; Wieland et al, 1987; Pelham, 1989). Thus, it would appear that any soluble protein in the lumen would be free to leave but the resident assembly proteins do not leave the ER, perhaps as a consequence of their function.

A commonly shared KDEL (Lys-Asp-Glu-Leu) carboxy-terminal sequence on resident ER proteins provided a clue to the mechanism of retention. Proteins bearing this KDEL sequence are bound by an integral membrane protein, the KDEL receptor (Munro and Pelham, 1986). When this sequence is removed, the truncated protein is slowly secreted, and when added onto a protein that is normally secreted, the protein is retained within the ER (Munro and Pelham, 1987). Thus, this sequence was both necessary and sufficient to ensure the retention of a protein in the ER.

Since the proteins that must be retained by this receptor are far more abundant than any static receptor within the ER could accomodate (Pelham, 1989, 1990; Vaux et al, 1990; Kelly, 1990), a salvage or retreival mechanism was proposed, whereby a KDEL receptor might function in a distal compartment to capture KDEL-terminating proteins leaving the ER with bulk flow of vesicular traffic. Proteins bearing this tetrapeptide signal can acquire Golgi-specific carbohydrate modifications indicating that the KDEL receptor is retreived from the cis-Golgi (Pelham, 1988; Dean and Pelham, 1990; Jackson et al, 1990; Peter et al, 1992). The receptor carries its ligands back to the ER where it releases them, then returns to the Golgi when unoccupied.

A homologous yeast HDEL-receptor was initially identified as the ERD2 gene product in *Saccharomyces cerevisiae* (Lewis et al, 1990; Semenza et al, 1990), and since homologues of this protein have been discovered in the yeast *Kluyveromyces lactis* (Lewis et al, 1990), the malarial parasite *Plasmodium falciparum* (Elmendorf and Haldar, 1993), *Drosophila*

melanogaster (Banfield and Pelham, manuscript in preparation), humans (two genes: Lewis and Pelham, 1990, 1992a; Hsu et al, 1992) and cows (Tang et al, 1993). *In vitro* binding assays have recently confirmed the ability of this protein to bind to KDEL and HDEL sequences (Wilson et al, 1993). Optimal binding occurs at acid pH suggesting that pH differences between organelles may facilitate binding and release of the ligand. Sequence analysis indicates conserved 7-transmembrane domains with only short loops in the cytoplasm and lumen. Mutational analysis (Townsley et al, 1993) showed that ligand binding is dependent on charged residues within the transmembrane domains. An aspartic acid residue in the last transmembrane domain is for transport of occupied receptor to the ER but not for presence in the Golgi, and thus receptor movement may be controlled by ligand binding (Lewis and Pelham, 1992b) along with conformational changes and intermolecular interactions within the membrane. Anti-idiotypic antibodies (monoclonal antibodies raised against antibodies) to carboxy-terminal KDEL sequences of two soluble, resident ER proteins recognized a quite different 72-kd putative mammalian KDEL receptor (Vaux et al, 1990). Further studies will be required to explain the striking size differences between these proteins and their roles.

E. Research Objectives

The control of breast cancer proliferation, based on early clinical data (Beatson, 1896) and rodent experimental models (Huggins et al, 1959), implicated a simple system essentially involving estrogens, progestins, and prolactin. Some of the estrogen regulation of breast tumor growth has been proposed to be mediated by the production of estromedins, or estrogen-induced mitogens (Sirbasku, 1978). IGF-II has been shown to be a putative growth stimulator in estrogen receptor (ER)-positive and ER-negative tumor epithelial cell lines. IGF-II mRNA transcripts present in the ER-positive T47D cell line, a MCF-7L subline, and an *in vivo* breast tumor xenograft were also estrogen inducible. In addition, two independent investigators found that MCF-7 clones transfected with preproIGF-II induced phenotypic alterations associated with malignant progression such as estrogen and

anchorage independent growth. These observations indicate that the estrogen stimulation of breast cancer cell proliferation *in vitro* and *in vivo* may be mediated by the production of IGF-II acting via an autocrine mechanism and this autocrine production of IGF-II may advance breast tumors to a more advanced estrogen independent stage. However, other investigators have observed IGF-II features not satisfied by a classical autocrine mode of action and suggest that IGF-II may act intracellularly. Cell proliferation by such an intracrine mechanism would explain the inablility of neutralizing antibodies against the growth factor to inhibit tumor cell growth. Since breast cancer therapy now requires an integration of multiple parameters, understanding the mechanism by which growth factors stimulate cell proliferation is essential for devising a successful therapeutic strategy.

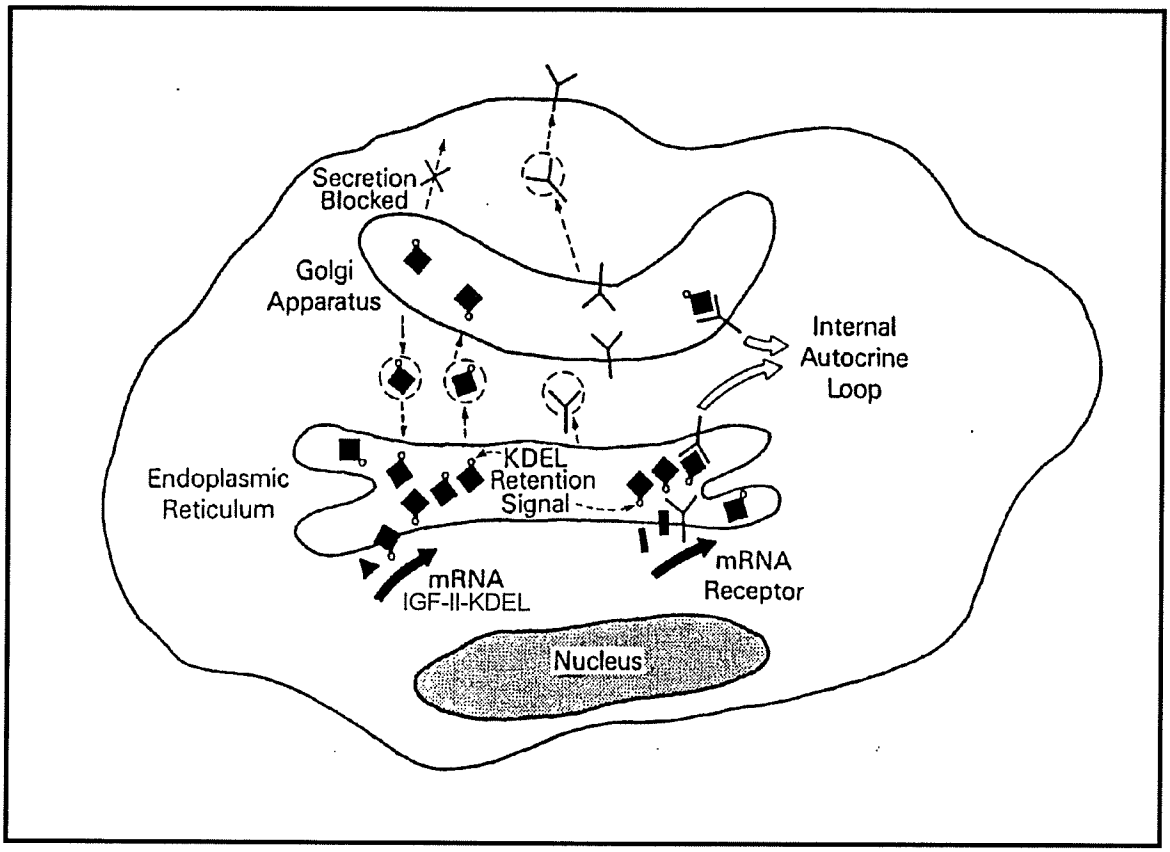
The approach used to determining whether IGF-II may act via an intracrine mechanism involves addressing the problem in three seperate stages:

1) Site-directed mutagenesis will be performed on the IGF-II cDNA in order to integrate a carboxyl terminus KDEL retention signal which prevents protein secretion (Figure 4).
2) MCF-7 clones transfected with this mutant IGF-II will be isolated.
3) Individual clones will then be characterized.

Fig. 4 ⇒

Figure 4. The cellular localization of IGF-II-KDEL following synthesis.

The addition of KDEL to the carboxyl terminus of soluble proteins results in their accumulation in the lumen of the ER. A receptor situated in early post-ER compartments recognizes the KDEL motif and captures such proteins containing the retention sequence and returns them to the ER. In these intracellular compartments, the ligand still has access to newly synthesized receptor routed to the cell surface and is insusceptible to inhibition by neutralizing antibodies.


Secretion
Blocked
Golgi
Apparatus
Internal
Autocrine
Loop
KDEL
Retention
Signal
Endoplasmic
Reticulum
mRNA
IGF-II-KDEL
mRNA
Receptor
Nucleus

MATERIALS AND METHODS

Site-directed Mutagenesis and Subcloning in the Expression Vector:

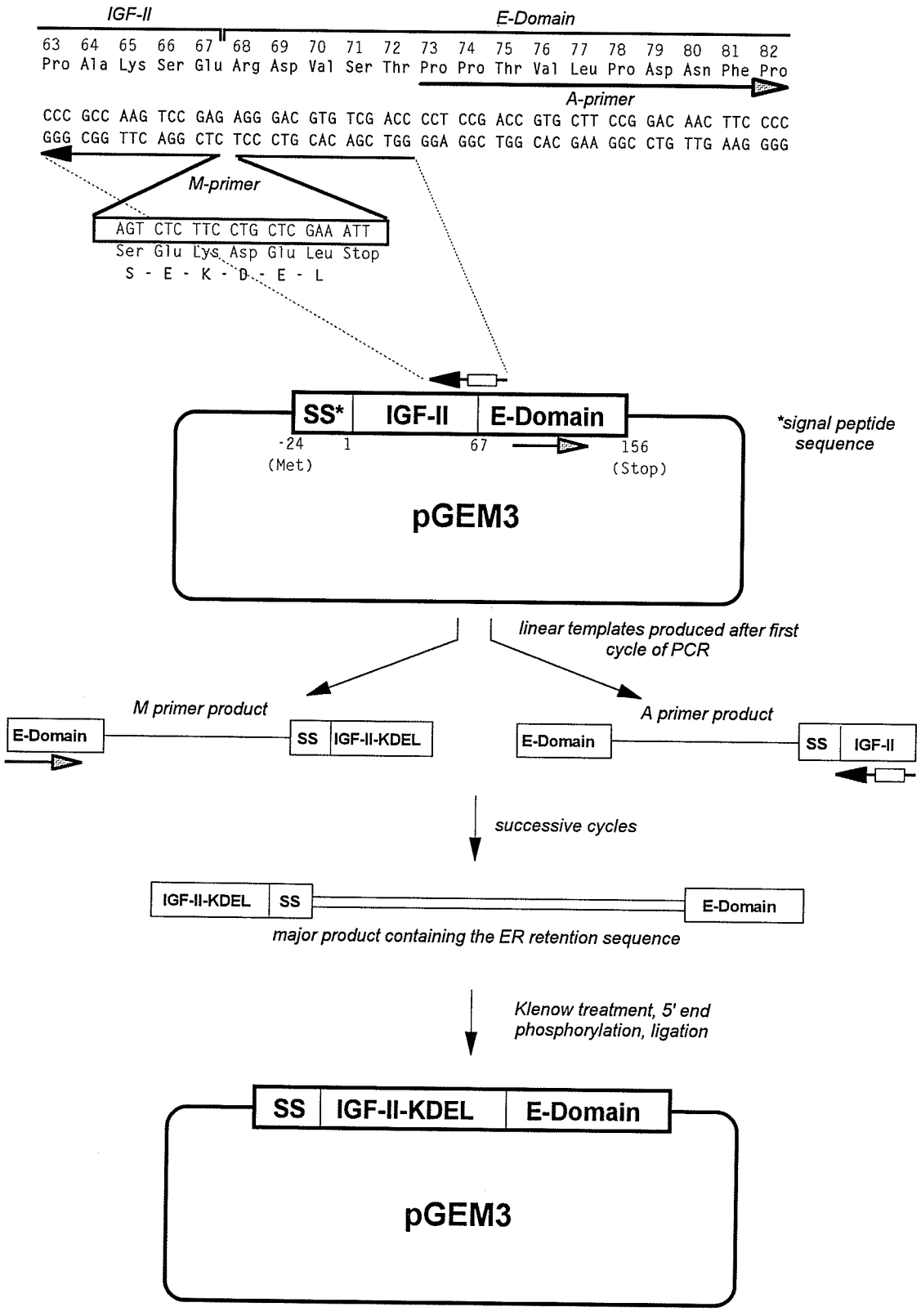
The 833-base pair *Pst*I fragment of cDNA encoding for human IGF-II preprohormone in pGEM3, generously provided by Dr. G.I. Bell (1985), was used as a template for manipulation by oligonucleotide-directed mutagenesis. PCR mediated site-directed mutagenesis of the IGF-II cDNA was based on a procedure described by Hemsley et al (1989). A 52 bp primer 3'-GGGCGGTTCAGGCTCAGTCTCTTCCTGCTCGAAATT TCCCTGCACAGCT**CC**-5' overlapping the intersection between the IGF-II, A and E domains, was used to strategically insert a 21 bp sequence encoding SEKDEL-STOP prior to the E domain (the last three base-T**CC** should have been T**GG**, but since this error came after the incorporated stop codon it did not effect the final translated product). The second primer 5'-CCTCCGACCGTGCTTCCGGACAACTTCCCC-3' lies adjacent to the 5' end of the previous primer on the opposing DNA strand (Figure 5). Template DNA was boiled in water and PCR buffer (Promega), then cooled quickly on ice before the addition of remaining components. A 50μl final reaction volume containing 2 ng of template DNA, 50 pmol of each primer, 200 μM of each dNTP (dATP, dCTP, dTTP, dGTP), 1X Promega PCR buffer and either 2 units of Taq or 1 unit of Vent DNA polymerase was incubated in a Pharmacia thermal cycler. The reaction mixture was initially heated at 98°C for 1.5 min to denature the DNA, cooled to 50°C for 2 min for primer annealing, then heated to 72°C for 4 min for elongation, for 3 cycles after which an additional two units of either Taq or Vent DNA polymerase was added. Additional 30 cycles began at 95°C for 1 min, then 60°C for 1.5 min, then finally 72°C for 3.5 min. The amplified PCR product was visualized on a 1% agarose gel following blunt ending with the Klenow fragment of *E. coli* DNA polymerase I in the PCR mixture.

The linear product was excised from agarose. It was 5' end phosphorylated with T4 Polynucleotide Kinase (Pharmacia), then blunt end ligated to itself using T4 DNA ligase (Pharmacia) to generate a circular plasmid, pGEM3-IGF-II-KDEL, with the desired

Fig. 5 ⇒

Figure 5. Strategy used for site directed mutagenesis.

PCR mediated site-directed mutagenesis of the IGF-II cDNA was based on a procedure described by Hemsley et al (1989) and is explained in more detail in MATERIALS AND METHODS. A 833-base pair *Pst*I fragment of cDNA encoding for the human preproIGF-II precursor, in the pGEM3 plasmid was used as a template for this manipulation. At top are the two primers used, their nucleotide sequences, and the location where they hybridize to in the IGF-II cDNA. The 52 bp M-primer hybridizes to the nucleotides encoding for amino acids 87 to 96 of preproIGF-II and overlaps the intersection between preIGF-II and the E domain. The center of the M-primer contained 21 extra nucleotides which form a loop upon hybridization and was thus used to strategically insert a 21 bp sequence encoding SEKDEL-stop into the IGF-II cDNA, prior to the E domain. The second primer, the A-primer, hybridized to the nucleotides encoding for amino acids 97 to 106 of the IGF-II precursor and lies adjacent to the 5' end of the M-primer on the opposing DNA strand. Since the primers are in a tail-to-tail orientation, PCR would amplify the entire plasmid. The first cycle of PCR generates linear products. The M-primer produces a linear product which has the KDEL sequence incorporated and is now used as a template for the A-primer. Similarly, the A-primer produces a product which is then used as a template for the M-primer. Successive cycles results in a reaction mixture which predominantly consists of a linear piece of DNA consisting of IGF-II-SEKDEL-stop cDNA sequence and pGEM3 plasmid. The linear product was then Klenow treated, 5' end phosphorylated, and blunt end ligated to itself to generate the starting circular plasmid with the desired additional bases incorporated.


IGF-II
E-Domain
63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82
Pro Ala Lys Ser Glu Arg Asp Val Ser Thr Pro Pro Thr Val Leu Pro Asp Asn Phe Pro
A-primer
CCC GCC AAG TCC GAG AGG GAC GTG TCG ACC CCT CCG ACC GTG CTT CCG GAC AAC TTC CCC
GGG CGG TTC AGG CTC TCC CTG CAC AGC TGG GGA GGC TGG CAC GAA GGC CTG TTG AAG GGG
M-primer
AGT CTC TTC CTG CTC GAA ATT
Ser Glu Lys Asp Glu Leu Stop
S - E - K - D - E - L
SS*
IGF-II
E-Domain
-24
(Met)
1
67
156
(Stop)
pGEM3
*signal peptide sequence
linear templates produced after first cycle of PCR
M primer product
E-Domain
SS
IGF-II-KDEL
A primer product
E-Domain
SS
IGF-II
successive cycles
IGF-II-KDEL
SS
E-Domain
major product containing the ER retention sequence
Klenow treatment, 5' end phosphorylation, ligation
SS
IGF-II-KDEL
E-Domain
pGEM3

additional bases incorporated. The mutation was confirmed by restriction endonuclease digestion using *Dra*I and nucleotide sequencing. This IGF-II-KDEL cDNA was excised from the pGEM3 plasmid using *Hin*dIII and *Xba*I restriction endonuclease enzymes, then subcloned into the corresponding restriction sites in the polylinker of pCMV4; a generous gift from DR. D.W. Russel (Andersson et al, 1989). Ligations were carried out overnight at 9°C followed by 15hr at 16°C. The resulting expression vector was designated CMV4-IGF-II-KDEL. A Simian virus 40 (SV40) promoter regulated expression vector, SV40-IGF-II-KDEL, was constructed by digesting a SV40CAT vector used in our lab with *Hpa*I and *Bgl*II to remove the CAT gene and inserting IGF-II-KDEL excised from the above CMV4-IGF-II-KDEL construct using *Sma*I and *Bgl*II. A metallothionein promoter regulated expression vector, MT-IGF-II-KDEL, was constructed by blunt-ending a *Bgl*II digested pPK9a vector (generously provided by Dr. A.H. Greenberg; Samuel et al, 1992) and inserting a blunt-ended IGF-II-KDEL cDNA. Restriction endonuclease analysis was used to determine that the insertion was correctly orientated.

DNA Sequencing:

Supercoiled plasmid DNA was converted to a single-stranded form prior to sequencing by alkali denaturation. In an eppendorf tube, 2μl of 2M NaOH/2mM EDTA was added to 3-4μg of plasmid DNA (in 20μl) and set aside at room temperature for 5 minutes. To the mixture was added 10μl 0.9M NaAc pH5.0 (3μl 3M NaAc and 7μl ddH_2O) to stop the reaction. Absolute ethanol (75μl) was added and DNA was then precipitated at -70°C for 5 minutes. DNA was recovered by centrifugation, dried and reconstituted in 7μl ddH_2O. The remaining procedure was carried out using DNA Sequencing with Sequenase kit from USB. Plasmid DNA, 2μl Sequenase Buffer (5×: 0.2M Tris-HCl pH 7.5, 0.1M $MgCl_2$, 0.25M NaCl), and 1μl primer (1pmol) were incubated in a 65°C water bath for 2min and cooled slowly to room temperature for primer to anneal. To the annealed template-primer was added 1μl DTT (0.1M), 2μl of 1:5 water diluted Labeling Mix (7.5μM dGTP, 7.5μM dCTP, 7.5μM dTTP), 0.5μl [α-^{35}S]dATP and 2μl of 1:8 Dilution

Buffer (10mM Tris-HCl pH 7.5, 5mM DTT, 0.5 mg/ml BSA) diluted Sequenase Enzyme. The elongation reaction was allowed to proceed slowly for 2-5 mins at room temp. In the mean time, four termination bases (ddGTP, ddATP, ddCTP, and ddTTP) were incubated in seperate eppendorf tubes at 37°C. A portion (3.5µl) of the reaction mixture was aliquoted to each termination codon and incubated at 37°C for 3-5 min. The reaction was stopped with 4µl of Stop Solution (95% formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF). The samples were boiled for 2-3 mins, cooled quickly on ice and immediately loaded onto a 6% polyacrylamide gel containing 7M urea. The gel was run using 1×Winter's TBE buffer for 2-5 hours at 1600 volts and 30mA.

Restriction Endonuclease Digestion and Agarose Gel Electrophoresis:

Typically, 2-3 units of enzyme was used per µg of DNA, in a 15-20µl reaction volume, with the manufactures recommended buffer accompanying the enzyme and at the appropriate temperature depending on the restriction enzyme used. The reaction was stopped 2-3hrs later with the addition of 1-2µl gel loading buffer (10× loading buffer: 0.46% Bromophenol Blue, 0.46% Xylene Cyanol, 50% Glycerol, 1mM EDTA), loaded onto a 1.2% agarose gel (in TBE buffer: 10.8g Tris base, 5.5g Boric acid, 10mM EDTA per litre pH8.0) containing ethidium bromide (0.5µg/ml) and electrophoresed in 1×TBE buffer at 20-120 volts for 1-12 hours (depending on size of gel, size of DNA, and voltage used). *Hin*dIII/ϕX digest standard DNA markers (Pharmacia) were prepared using reaction buffer conditions identical to, and electrophoresed in parallel with, the DNA samples. DNA was visualized by placing the gel on ultraviolet light transilluminator and photographed.

Southern Transfer:

Following electrophoreses, one corner of the gel was cut in order to orient the gel during succeeding operations. DNA was denatured by soaking gel in 1.5M NaCl/0.5 NaOH with

gentle shaking for one hour at room temperature, and neutralized in 1.5M NaCl/1M Tris (pH8.0) under the same conditions. The procedure for transfering DNA to nitrocellulose was similar to that outlined in Maniatis et al (1982). Briefly, the gel was placed upside down on a 20×SSC (3M NaCl, 0.3M sodium citrate pH7.0) soaked Whatman 3MM paper on a glass tray with the Whatman paper overlapping into 20×SSC solution. The gel was clipped at one corner for orientation, covered by the same size piece of nitrocellulose membrane (nitroplus 2000 Micon Separations Inc.) which was soaked for 10-15 seconds in water (~80°C) and then covered by two same size pieces of Whatman 3MM paper soaked in 20×SSC solution. Any bubbles during the procedure were removed by rolling with a glass pipette. Plastic wrap placed around the gel to prevent tranfer solution from evaporating. Paper towels were put on the gel-nitrocellulose-Whatman paper stack, covered by a weight (~1kg), and allowed to transfer for 12-16hrs. The nitrocellulose was then rinsed in 6× SSC for 5 minutes, air dried, and baked for 2hrs at 80°C.

DNA labelling and Hybridization:

The nitrocellulose filter was placed in a plastic bag. About 10ml of prehybridization solution (50% formamide-1× Denhardt's-0.1% sodium dodecyl sulfate-100 μg/ml denatured salmon sperm DNA-5×SSC) was added to the bag, air bubbles were removed, the bag was sealed using a Quick-seal bag sealer (National Instrument Company, Inc.), and placed in a 42°C water bath while the radioactive probe was being prepared. Hybridization using ~3-5 ng/ml of ^{32}P-labelled complementary probe was carried out in the same prehybridization solution for 24h at 42°C. Blots were washed to a final stringency of 0.1×SSC (1×SSC: 0.015 M Sodium citrate, 0.15 M NaCl, pH 7.0) at 65°C for 20 min, and exposed to Kodak XAR X-ray film at -70°C using an intensifying screen. All autoradiograms were scanned with a SCANPLOT version 4.01 software combined densitometric system.

DNA probe was ^{32}P-labelled by a random priming kit from Pharmacia using T7 polymerase and [^{32}P]-α-dCTP. Briefly, the doubled stranded DNA was boiled, cooled

quickly on ice, and annealed to random oligonucleotide primers. Primers were then elongated by T7 polymerase in the presence of dATP, dTTP, dGTP and radioactive dCTP at 37°C for 5-10 min. The product was seperated from free radioactive nucleotide by using a Sepharose G-50 column, denatured by boiling and added to the bag with the prehybridized filter.

Competent Cells and Transformation:

A sterile loop was used to innoculate *E. coli* bacteria from glycerol stock into 5 ml of LB broth (1 litre LB broth: 10g bactotryptone, 5g yeast, 10g NaCl, pH 7.5) which was grown overnight in a shaking incubator (250 RPM) at 37°C. The following morning 1 ml of the over-night culture was added to 100 ml of LB broth in a large sterile flask and once again incubated for ~1.5hr to a density of $\sim 5 \times 10^7$ cells/ml (OD_{550}= 0.5). The remaining procedures were performed on ice or at 4°C. The culture was divided into two 50ml centrifuge tubes, pelleted by centrifugation at 4000 × g for 5 min, resuspended gently in 33.3ml (1/3 of original volume) of a freshly prepared 0.1M CaCl chilled solution with the aid of a 10ml pipette, and set on ice in the cold box for 4hr. This solution was pelleted as above and resuspended in 6.7ml (1/15 of original volume) of a fresh 0.1M CaCl/15% glycerol chilled solution. 200µl were aliquoted into eppendorf tubes and stored at -70°C until required for transformation. Efficiency of transformation increased with the length of time cells were stored.

Transformation was performed by adding 3-6µl of plasmid (~10ng) or the ligation mixture to 70µl of competent cells and set on ice for 30 min. Cells were then heat shocked with gently agitation in a 42°C for 1.5 min, after which 200µl of LB was added and cells were allowed to recover at 37°C for 30 min. A range of 50-200µl were spread on LB agar plates (1.2g in 100ml LB broth) containing 50 µg/ml ampicillin and incubated upside down at 37°C overnight.

Plasmid DNA Amplification and Isolation:

Following bacterial amplification, plasmids were isolated from transformed bacteria and purified by CsCl density gradient centrifugation as described by Maniatis et al (1982). A transformed bacterial colony was picked with a sterile loop, innoculated into 5 ml LB broth containing 50 μg/ml ampicillin and incubated in a shaker incubator for a minimum of 8hr. At this point, a small scale preparation of DNA (Minipreps) was performed with 1-1.5ml of this culture using a one step protocol described by Chowdhury (1991) or by using Magic Minipreps (Promega). The isolated plasmid DNA pellet was reconstituted in TE buffer, digested with the appropriate restriction enzyme and electrophoresed on a 1.2% agarose gel. After confirming that the appropriate ligation product was obtained (or that the correct plasmid was amplified), the remaining bacterial culture was added to a large flask containg either 500ml or 1 liter of LB broth with 50 μg/ml ampicillin and incubated overnight.

The bacterial culture was centrifuged in two 500ml Beckman tubes in a JA10 rotor for 8 min at 6000 rpm and 4°C. The supernatent was discarded and cells were resuspended in 9.5ml of cold lysis buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50mM glucose). After transfering to 40ml Oakridge tubes, 500 μl of freshly prepared chicken egg white lysozyme (20 mg/ml in lysis buffer) was added and cells were shaken on ice for 30 min. 10 mls of a 0.2M NaOH/0.2% SDS solution was then added, followed by 10 mls of a 3M KAc solution (pH 5), after which cells were again incubated with shaking for 30 min following additional of each solution. Bacterial debris was removed by centrifugation at 18,000 rpm (4°C) for 30 min and supernatant was then transfered to 50ml centrifuge tubes. Following extraction with 10-15 ml of warm (65°C) phenol : chloroform : isoamyl alcohol (25:24:1) the (top) aqueous phase was removed. DNA was precipitated by the addition of 0.6 volumes of isopropanol and allowed to sit at room temperature for 15 min before recovering by centrifugation in a JA20 rotor at 15,000 rpm for 10 min using Corex tubes. The DNA was resuspended in 6 mls of TE buffer. 6.6 g of CsCl was added and the dissolved mixture was transfered to a Beckman Quickseal Ti75 centrifuge tube. EtBr

(0.2 ml of a 1mg/ml solution) was overlayed and the remaining volume was filled with mineral oil. The tubes were balanced, heat sealed, and centrifuged in a Beckman Ti75 rotor at 55,000 rpm for 16hr, then at 45,000 rpm for 45 min (both speeds were at 25°C). DNA was visualized with UV light. The plasmid band was removed carefully with an 18-gauge needle and 3 ml syringe without disturbing the upper bacterial genomic DNA. EtBr was removed by 2-3 extractions with 5mls of isoamyl alcohol. Plasmid DNA was precipitated with 2.2 volumes of ethanol (-70°C), centrifuged for 30 min at 15,000 rpm in a JA20 rotor, dried, and then reconstituted in ddH_2O. DNA was quantitated spectrophometrically by measuring the absorbance at 260 nm.

Transfection and Cell Culture:

The MCF-7 human breast cancer cell line was maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were seeded at a density of $7.5x10^5$ cells per 10cm tissue culture dish, two days prior to infection, in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% (v/v) fetal calf serum, L-glutamine (4mM), glucose (4.5 g/liter), and antibiotics penicillin and streptomycin (100 IU/ml and 100 μg/ml, respectively). Cells were approximately 50% confluent following incubation for 48 h. Cells were washed 7 ml of serum free medium was added before calcium phosphate coprecipitation with a vector containing a gene for neomycin resistance from Dr. P. Cattinni (Department of Physiology, University of Manitoba) designated pNEO-DX. Calcium phosphate ($CaPO_4$)-DNA precipitates of 15 μg CMV4-IGF-II-KDEL, SV40-IGF-II-KDEL, or MET-IGF-II-KDEL, and 5 μg pNEO DX; 15 μg pCMV and 5 μg pNEO-DX; and 5 μg pNEO-DX alone were added to individual plates for 4 hours, then glycerol shocked with 20% glycerol in DMEM for 8 min to facilitate DNA uptake. Cells were finally rinsed twice with serum free medium and allowed to recover for 24 h in 10% FCS under conditions mentioned above. Stable cells were selected in 10% FCS containing 0.8 mg/ml of a neomycin analog. G418. After approximately two weeks single clones were isolated with cloning rings, and passaged into a 24 well plate, then into 6 well plates, then into 10 cm plates. Clones surviving the initial selection and passage into

a 24 well plate usually survived the entire cloning process. G418 (0.5 mg/ml) was added to medium up until clones were initially analyzed for IGF-II transcript and stock cells were frozen. All clones positive for IGF-II-KDEL mRNA were preserved with 7.5% DMSO-10% FBS in liquid N_2.

RNA Isolation and Northern Blot Analysis:

Total cellular RNA was extracted from cell monolayers by homogenizing in a guanidinium thiocyanate solution (GuSCN: 4M guanidine thiocyanate, 17 mM N-lauroyl sarcosine, 0.007% 2-mercaptoethanol, and 25 mM sodium citrate pH7.0), aspirated through a 21 gauge needle in order to sufficiently break up the cells, layed over a 5ml cesium chloride cushion (5.7M cesium chloride, 0.1 M EDTA) in Beckman Quickseal centrifuge tubes, and then centrifuged at 35000 rpm for 18hrs in a Ti75 rotor as described by Chirgwin et al (1979). The pellet, following centrifugation, was resuspended in ddH_2O, precipitated with sodium acetate-ethanol (0.2M NaAc, 2.2 volumes ethanol) at -70°C and once again pelleted, dried, and reconstituted in ddH_2O. RNA was quantitated spectrophotometrically at a wavelength of 260nm.

25µg of total RNA per sample (in a ~10µl sample volume) was denatured by adding 10µl formamide, 4.7µl formaldehyde, 2.7µl of 5× gel running buffer (GRB: 40mM MOPS pH 7.0, 10 mM sodium acetate pH 5.2, 1mM EDTA pH 8.0), 3µl loading buffer upto a volume of 30µl and incubated at 65°C for 5min. The denatured RNA was electrophoresed on a 1% agarose-2.2M formaldehyde denaturing gel containing ethidium bromide (0.5µg/ml) emersed in 1× GRB as outlined in Maniatis et al (1982) and transfered to a nitroplus 2000 (Micon Separations Inc.) membrane. The procedure for blotting RNA from formaldehyde to nitrocellulose was identical to that of southern transfer, with the removal of denaturing washes followed by neutralization, since RNA is single stranded. Blots were washed to a final stringency of 0.1×SSC (1×SSC: 0.015 M Sodium citrate, 0.15 M NaCl, pH 7.0) at 65°C for 20 min, and exposed to Kodak XAR X-ray film at -70°C using an intensifying screen. To control for variation in RNA loading and transfer,

blots were first stripped of IGF-II probe, then rehybridized with a human ^{32}P-labeled GAPDH probe.

DNA Isolation and Southern Blot Analysis:

Genomic DNA was isolated using a protocol described by Davis et al (1986) from frozen cell pellets. Cells were washed with Phosphate Buffered Saline (PBS: 8.0g NaCl, 0.2g KCl, 1.5g NaH_2PO_4, 0.2g K_2HPO_4 per litre of solution), scraped from a 15cm cell culture plates using a rubber policeman, then washed again with PBS before being pelleted and stored at -70°C. The cell pellet was resuspended in 10ml of TE buffer (10mM Tris-HCl, 0.1 M EDTA, pH 7.4). SDS was mixed in gently to a final concentration of 100µg/ml, mixed well and incubated at 37°C for 4 h (for protein digestion). The protein digested mixture was then transfered to a 60°C water bath. RNase was added to a final concentration of 50µg/ml. The Genomic DNA was purified with two phenol/chloroform extractions followed by a single extraction with 24:1 chloroform:isoamyl alcohol. DNA was precipitated with 0.2 M NaAcetate and 2.2 volumes of ethanol, dried and resuspended in ddH_2O.

Southern analysis was performed on 0.8% agarose gels following digestion with *Pst*I restriction endonuclease. Typically 10µg of DNA was digested overnight with 30 units of *Pst*I restriction enzyme, then 30 units of additional *Pst*I enzyme for a further 6 hrs. Digested DNA was size-seperated by electrophoresis on a 0.8% agarose gel (in 1× TBE) overnight at 20-30 volts. Southern transfer of genomic DNA was the same as described above, with slight changes. The fractionated genomic DNA was denatured in a 0.5M NaOH-1.5M NaCl solution for 30 min with gentle agitation and neutralized in 1M NH_4 Ac-20mM NaOH for another 30 min. This same neutralizing solution was used to transfer genomic DNA onto a nitrocellulose membrane (minimum 14 hrs transfer time). Baked blots were prehybridized in 50% Formamide/SCP (20×SCP: 2M NaCl, 0.6M Na_2HPO_4, 0.02M EDTA, pH 6.2) prehybridization solution (50% formamide, 6.6×SCP, 0.4% N-lauryl sarcosine, 4×Denhardt's, 200µg/ml Sheared Salmon Sperm DNA).

Hybridization was carried out in the same prehybridization solution for 18-36hrs. Blots were washed using SCP containing 1% sarcosyl to a final stringency of 0.1×SCP/1% sarcosyl at 65°C for 30 min.

Extraction of IGF-II from cells and conditioned medium:

Cells were cultured to 80% confluence (sample 1) or to confluence (sample 2) in 6-well plates in 5%-FBS. The media was removed and cells were washed three times with phosphate-buffered saline (PBS: 8.0g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, per litre) to remove residual serum, then lysed with 500µl of freshly prepared, chilled lysis bufer (3.3M formic acid-0.5% Tween-20). The lysis buffer and extraction procedure used to isolate IGF-II from tissue culture cells are as described by Lee et al (1991). The extracts were diluted 1:20 or 1:40 with RIA buffer (50mM Na phosphate-0.1% NaCl-0.1% EDTA-0.1% Na azide-0.02% protamine sulfate-0.05% Tween-20, pH 7.5) before RIA.

For collection of conditioned media, 8×10^5 cells were seeded in 5%-FBS on 10 cm^2 tissue culture plates. On the following morning, three washes were done with PBS and cells were maintained in 10ml SF media (Ham's F12 and Dulbecco's modified Eagle's medium (1:1), 2.2g/l sodium bicarbonate, 15mM PIPES buffer, 10µg/ml human transferrin) for three days (72 hrs). Conditioned media was collected, centrifuged to remove debris and stored at -70°C. A nearly identical extraction procedure used to isolate IGF-II from cell culture was employed to enrich IGF-II from conditioned medium (Bowsher et al, 1991).

IGF-II Radioimmunoassay:

A human IGF-II RIA described by Bowsher et al (1991) and Lee et al (1991) was implemented by Helle Cosby using an anti-IGF-II monoclonal antibody (generously provided by Dr. K. Nishikawa). A rabbit antimouse IgG antibody was used for the secondary antibody reaction and was diluted 1:100 with RIA buffer containing 0.2% gelatin.

Cell Growth Studies and the MTT Assay:

Cells were detached from stock flasks with trypsin/EDTA in Hank's balanced salt solution (GIBCO laboratories). Trypsin was neutralized by the addition of 5% csFBS. Cells were then washed by centrifugation at 1000 rpm for 2 min then resuspending in serum free (SF) medium as described by Karey and Sirbasku (1988; Ham's F12 and Dulbecco's modified Eagle's medium (1:1), 2.2g/l sodium bicarbonate, 15mM PIPES buffer, 10μg/ml human transferrin). This washing was performed thrice to remove traces of FBS. Another set was resuspended and plated in 5%csFBS. Both sets of cells were then dispersed by gently syringing with a 16 gauge needle, counted using a Coulter counter, and 1×10^4 cells (in 200μl of either SF or 5%csFBS) were plated (in triplicate) in five 96-well plates. On the following morning 5%csFBS was remove very carefully using a pasteur pipette and a rubber bulb, cells were (very carefully) washed thrice and replaced with SF medium. Medium was changed every 3 days.

Cells were plated the same as described above when assayed for response to exogenous IGF-II. Recombinant IGF-II (Amersham) was diluted in SF-medium and added to cells 24hrs after plating at a concentrations of 5 and 25μg/ml. More IGF-II was supplied with fresh medium every 3 days.

Cell number was determined using a colorimetric assay using MTT. This assay measures the reduction of tetrazolium salt (MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical) by living cells to a blue-colored formazan product. The cell viability assay was performed as described previously (Mosmann, 1983; Alley et al, 1988; Watson et al, 1991). MTT assay was performed on days 1 (one day after plating), 5, 9, 12, and 15. On the morning of the assay, cells were incubated for 6h at 37°C in 100μl of fresh SF medium plus 10μl of water dissolved tetrazolium salt (MTT; Sigma). Stop buffer was added (190μl of (1:1) isopropanol/water made 0.4M in HCl) to each well and contents were vigorously mixed by pipeting in order to dissolve the reduced MTT granules. Absorption was measured at A_{590} with a Biotec ELISA Plate reader.

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RESULTS

Construction of an IGF-II cDNA containing a KDEL ER Retention Sequence and its Expression in MCF-7 Cells.

In order to determine whether IGF-II could act by an intracrine mechanism, a slightly modified version of the inverse PCR protocol ascribed by Hemsley et al (1989) was used to introduce the nucleotide sequence encoding for the Lys-Asp-Glu-Leu (KDEL) endoplasmic reticulum retention signal into the IGF-II cDNA. The insertion was made just preceding the E-domain at the 3' end of the sequence for the 67 amino acid biologically active IGF-II peptide. The KDEL sequence was not placed at the carboxyl terminus of the pre-pro-IGF-II precursor molecule since processing after synthesis would result in the retention sequence residing in the cleaved E peptide and not in the IGF-II molecule (see Figure 2). It was, therefore, also necessary to include a stop codon following the KDEL sequence so that the E-domain would not be made. The amplified product was recircularized by ligation (see MATERIALS AND METHODS). A summary of the cloning strategy is depicted in Figure 5. Following site-directed mutagenesis an additional *Dra*I endonuclease restriction site was introduced into the newly generated IGF-II-KDEL cDNA. Restriction digestion of the mutagenized cDNA using *Dra*I confirmed that the desired sequence had been incorporated.

Taq DNA polymerase is known to introduce base-pair mismatches (Saiki et al, 1988). Sequence analysis confirmed that the insertion had occured correctly and no other alterations within the coding sequence had occured. A few bases were missing at the junction between the PCR primers, where the ligation had occured. The deletion was likely the consequence of two basepair mismatches (CC instead of GG) that were inadvertently introduced at the 5' end of the mutant primer. Since this error followed the newly incorporated stop codon, it did not affect the final translated product.

This mutated IGF-II-KDEL cDNA was then subcloned into three different expression

vectors in order to produce clones with varying and regulatable levels of IGF-II expression. Messenger RNA synthesis from these vectors was directed by a CMV strong constitutive promoter (pCMV4), a weaker SV40 promoter (pSV40), or a metallothionein inducible promoter (pMT), and the newly constructed vectors were designated CMV-IGF-II-KDEL, SV40-IGF-II-KDEL, and MT-IGF-II-KDEL, respectively. Three sets of transfections were performed with: i) CMV-IGF-II-KDEL, SV40-IGF-II-KDEL, or MT-IGF-II-KDEL, plus a vector containing a cDNA encoding for neomycin resistance (pNEO-DX); ii) pCMV4 without insert plus pNEO-DX, as a control, designated CMV-NEO; and, iii) pNEO-DX alone also as a control, designated NEO, into wild type MCF-7 human breast cancer cells. Stable clones were selected for their ability to grow in the presence of a neomycin analog, G418. Following transfection with the CMV-IGF-II-KDEL expression vector, many more single colonies (~5-10×) survived the initial G418 selection than those transfected with either the SV40-IGF-II-KDEL, MT-IGF-II-KDEL, or CMV-NEO and NEO control vectors. All transfections were performed under identical conditions using equal amounts of a pNEO DX neomycin resistance vector with the only difference being the expression vector used.

Cloning cylinders were then used to isolate the stable colonies of transfected cells, which were allowed to expand by passaging to larger and larger dishes. During early stages of selection, 15%FBS in MCF-7 conditioned medium was used to assist growth. The first passage involving the transfer of the G418-resistant colonies into a 24-well dish using a silicone gel sealed cloning cylinder was critical for clones to continue growing. This first step was executed very carefully because cells surviving this first stage of selection did not have trouble during successive steps. Cells were maintained in G418 containing medium throughout their growth expansion.

Of the twenty-five CMV-IGF-II-KDEL colonies isolated, twelve survived the cloning process. The very few MT-IGF-II-KDEL colonies did not survive the first passage and none of the three SV40-IGF-II-KDEL clones attained produced the IGF-II message. Several CMV-IGF-II-KDEL clones were chosen for extensive characterization. In

addition, stable clones transfected with CMV-NEO and NEO were used as controls.

Stable clones were then screened for production of the mutated IGF-II transcript by Northern blot hybridization and seven of the twelve CMV-IGF-II-KDEL clones were found to produce the mutant IGF-II transcript (Figure 6). One prominent 1.6kb RNA species was seen in clones 3, 8, 12, 15, 23, 24, and 25, when 25μg of total RNA from each clone on a Northern blot was hybridized with an ^{32}P-IGF-II cDNA probe. Blots were then hybridized with a GAPDH probe in order to control for loading differences. Densitometry was used to quantitatively varify these loading differences. A range of IGF-II-KDEL expression was established. This 1.6kb band is in agreement with the transcript size expected from the CMV-IGF-II-KDEL expression vector. Clones 1, 2, 11, 19, and 20 did not produce detectable IGF-II mRNA. IGF-II transcript was also undetectable in CMV-NEO and NEO clones, or wild type MCF-7 cells, which is in agreement with other investigators who had failed to detect IGF-II expression in the MCF-7 human breast cancer cell line (Furlanetto and DiCarlo, 1984; Yee et al, 1988; Darbre and Daly, 1990). Since endogenous IGF-II would interfere with our hypothesis, a non-expressing cell line was essential in selecting a model system to test whether IGF-II could act via an intracrine pathway. Thus, only the one 1.6kb mRNA species was observed in successfully transfected CMV-IGF-II-KDEL clones.

Genomic Southern hybridization experiments were performed in order to determine whether the differences in the levels of IGF-II expression in our clones were the result of different number of vector DNA incorporated into the cellular genome. Genomic DNA was isolated from all established clones and digested with the restriction endonuclease, *Pst* I. An 833 base pair band, which was easily distinguishable from the endogenous IGF-II genes band pattern, was present in all clones expressing IGF-II mRNA (Figure 7). No correlation was evident between integrated copy number and the level of expression, which we would expect since the level of expression is highly dependent on the sight of integration in the genome. This 833 base pair fragment was also present in CMV-IGF-II-KDEL clone 19, but no IGF-II transcript was detected.

Fig. 6 ⇒

Figure 6. Northern blot analysis of IGF-II mRNA expression in MCF-7 clones.

Total RNA (25μg) was analyzed from MCF-7 clones transfected with, the pCMV4-IGF-II-KDEL vector (lanes marked CMV-IGF-II-KDEL CLONES 3, 8, 12, 15, 24, 23, and 25) which showed varying levels of mRNA expression that is quantitated below using densitometry, and the pCMV4 parent vector without insert (lanes marked CMV-NEO 1 and 2) which shows the negative control profile of wild-type MCF-7 cells. Also included is 10μg of total mRNA from BRL cells as a positive control. Blots were hybridized with a ^{32}P-labeled IGF-II complementary DNA probe. To control for variation in RNA loading and transfer, blots were first stripped of IGF-II probe, then rehybridized with a human ^{32}P-labeled GAPDH probe (shown below).

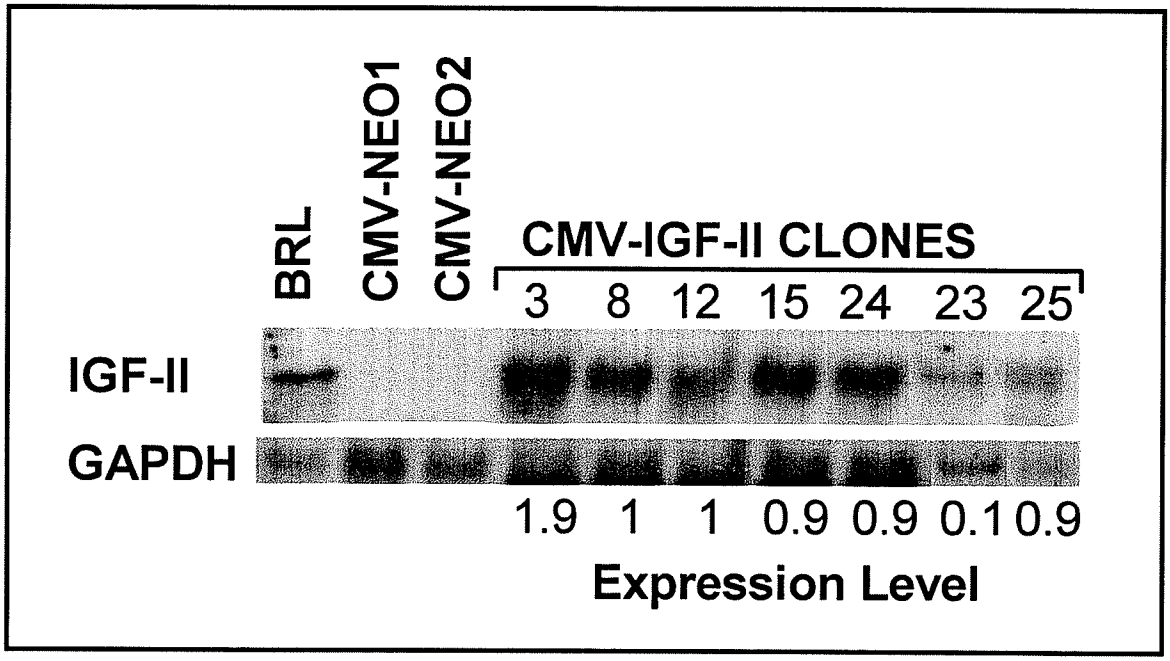
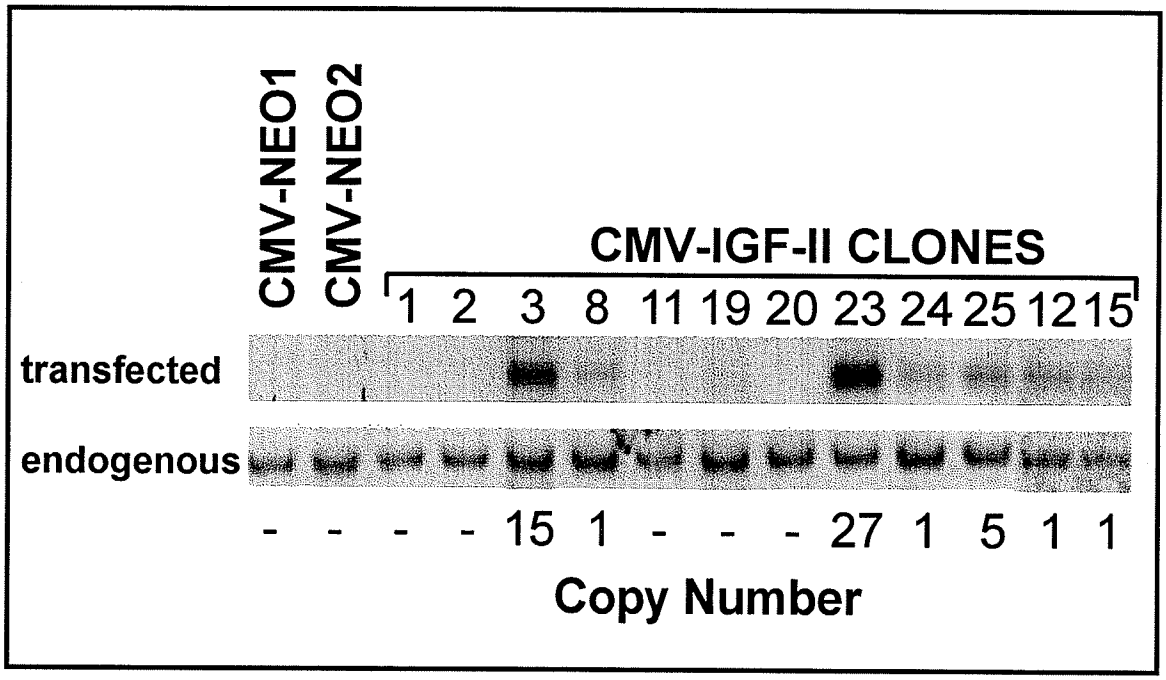

BRL
CMV-NEO1
CMV-NEO2
CMV-IGF-II CLONES
3 8 12 15 24 23 25
IGF-II
GAPDH
1.9 1 1 0.9 0.9 0.1 0.9
Expression Level

Fig. 7 ⟹

Figure 7. Southern blot analysis of IGF-II copy number.

Southern analysis was performed with 10μg of *Pst*I digested genomic DNA from all MCF-7 clones and hybridized with a ^{32}P-labeled IGF-II cDNA probe. An 833 base pair band, which was easily distinguishable from the endogenous IGF-II genes band pattern, was present in successfully transfected cells. The copy number of IGF-II incorporated into the cellular genome after transfection was determined using densitometric scanning. Quantitation was done relative to the endogenous IGF-II gene.


CMV-NEO1
CMV-NEO2
CMV-IGF-II CLONES
1 2 3 8 11 19 20 23 24 25 12 15
transfected
endogenous
- - - - 15 1 - - - 27 1 5 1 1
Copy Number

IGF-II Radioimmunoassay of Cellular Extracts and Conditioned Media.

An extraction procedure described by Lee et al (1991) was performed on several clones to verify that clones expressing IGF-II transcript were also translating this mRNA to produce the corresponding IGF-II-KDEL intracellular protein product. Since significantly higher RIA values were attained from cellular extracts of our clones that produce the IGF-II-KDEL transcript than what would be expected from a normally secreted protein, this indicated that the KDEL sequence was causing the accumulation of IGF-II inside the cell (Table 1). It appears that cell density may have influenced the amount of IGF-II-KDEL expression. Protein extractions were done on cells in Sample 1 at 80% confluence, while cells in Sample 2 were at confluence. The absolute protein values are higher in Sample 1 but fold over clone 12 values are much more similar (see Table 1). A correlation between intracellular IGF-II protein and IGF-II mRNA levels was not evident (Table 2). Variations may be the result of differences in mRNA or protein stabilities from one clone to another.

The tetrapeptide KDEL sequence at the carboxyl terminus of soluble proteins was shown to be necessary and sufficient for retention of such proteins within the lumen of the ER and prevents their secretion (Munro and Pelham, 1987; Pelham, 1989). Other investigators have found that a strong promoter may result in slight leakage of KDEL-containing protein into the medium (Rose and Doms, 1988; Dunbar et al, 1989; Haugejorden et al, 1991; Rose-John et al, 1993). To determine whether the presence of the ER retention sequence was preventing IGF-II secretion, conditioned media from CMV-IGF-II-KDEL clones, control CMV-NEO and NEO clones, and MCF-7 cells were analyzed for secreted IGF-II activity by radioimmunoassay. Since breast cancer cell lines secrete high levels of IGF binding proteins (De Leon et al, 1989; Clemmons et al, 1990; Owens et al, 1993), which could interfere with this assay, a very similar extraction procedure (Bowsher et al, 1991) as the one employed for cellular proteins above was used to isolate IGF-II. No detectable immunoreactivity was present in concentrated serum free medium conditioned by clones for two days, indicating that the KDEL retention signal

Table 1: Cellular IGF-II Levels determined by RIA.

Cells	SAMPLE 1 (ng/mg protein, N=4)	fold	SAMPLE 2 (ng/mg protein, N=3)	fold
MCF-7 wild type	---		---	
CMV-Neo	---		---	
CMV-IGF-II-KDEL clone 3	452 ± 160	2.7	111 ± 78	3.7
CMV-IGF-II-KDEL clone 8	582 ± 227	3.4	114 ± 12	3.8
CMV-IGF-II-KDEL clone 12	170 ± 43	1.0	30 ± 7	1.0
CMV-IGF-II-KDEL clone 15	nd	nd	143 ± 42	4.8
CMV-IGF-II-KDEL clone 24	375 ± 114	2.2	91 ± 2	3.0
Buffalo rat liver BRL-3A	84 (N=1)			

The protein extraction and RIA procedures, as well as, monoclonal antibody used are outlined in Materials and Methods. Immunoreactive IGF-II concentrations are derived from assay of each cell lysate preparation at 80% confluence (SAMPLE 1) or at confluence (SAMPLE 2). The absolute RIA values are after background MCF-7 wild type and CMV-NEO clones were subtracted. Fold values are relative to clone 12. (nd, not determined).

Table 2: Summary of Southern, Northern and Protein (RIA) values.

IGF-II	Clone 3	Clone 8	Clone 12	Clone 15	Clone 23	Clone 24	Clone 25
Exogenous Gene Copy	15	1	1	1	27	1	5
mRNA	1.9	1	1	0.9	0.1	0.9	0.9
Protein	2.7	3.4	1	nd	nd	2.2	nd

was working effectively.

Effect of Intracellular IGF-II Retention on MCF-7 Breast Cancer Cell Proliferation.

We next examined if intracellular IGF-II effects cell proliferation. MCF-7 wild type cells, CMV-IGF-II-KDEL and control clones were plated and grown in serum-free (SF) medium described by Karey and Sirbasku (1988). Because FBS is necessary for proper adhesion of cells (Arteaga and Osborne, 1989), therefore, in parallel, cells were also plated in 5% csFBS for 24 hours to facilitate adhesion then gently washed before receiving serum-free medium. Charcoal stripped FBS was used instead of regular FBS in order to reduce the amount of residual steroid hormones that may be left after washing. All plates received fresh medium every three to four days. Cell number was determined with the colorimetric assay using MTT, which measures the reduction of tetrazolium salt by living cells to a blue-colored formazan product. The MTT cell viability assay was performed 24 hours after plating and indicated that all clones had similar adhesive abilities, with cells plated in csFCS adhering slightly better then cells plated in SF medium (Figure 8). Inspite of this small difference in adhesion, the growth properties remained very similar during the growth assay (Figures 9 and 10). All IGF-II-KDEL expressing clones mimicked the slow growth behavior of control cells, with the exception of clone 12. Interestingly, clone 12 grew quite happily to high density without any serum. This growth could not be attributed to IGF-II-KDEL production since four other IGF-II-KDEL expressing clones display growth comparable to control cells.

Effect of Exogenous IGF-II on MCF-7 wild-type cells and CMV-IGF-II clones.

IGF-II has been shown by several groups to be potent for breast cancer epithelial cells and, in particularly, MCF-7 cells (Furlanetto and DiCarlo, 1984; Myal et al, 1984; Yee et al, 1988; Karey and Sirbasku, 1988; Osborne et al, 1989; Daly et al, 1991). Recombinant IGF-II at concentrations of 5 and 25ng/ml significantly increased the monolayer growth of our MCF-7 cells in SF medium (Figure 11a). IGF-II responsiveness

Fig. 8 ⇒

Figure 8. Adhesion properties of MCF-7 clones.

MCF-7 wild type cells, CMV-IGF-II-KDEL clones (clone 3, 8, 12, 15, and 24) and control clones (CMV-NEO 1 and 2, and NEO) were plated at 1×10^4 cells per 96-well plate in the serum-free medium and in 5% csFBS, in parallel. On the following morning, the medium and cells in suspension were removed, fresh serum-free medium was added, and the cells adhereing to the cell surface were assayed using the MTT assay. Absorbance was measured at A_{590}.

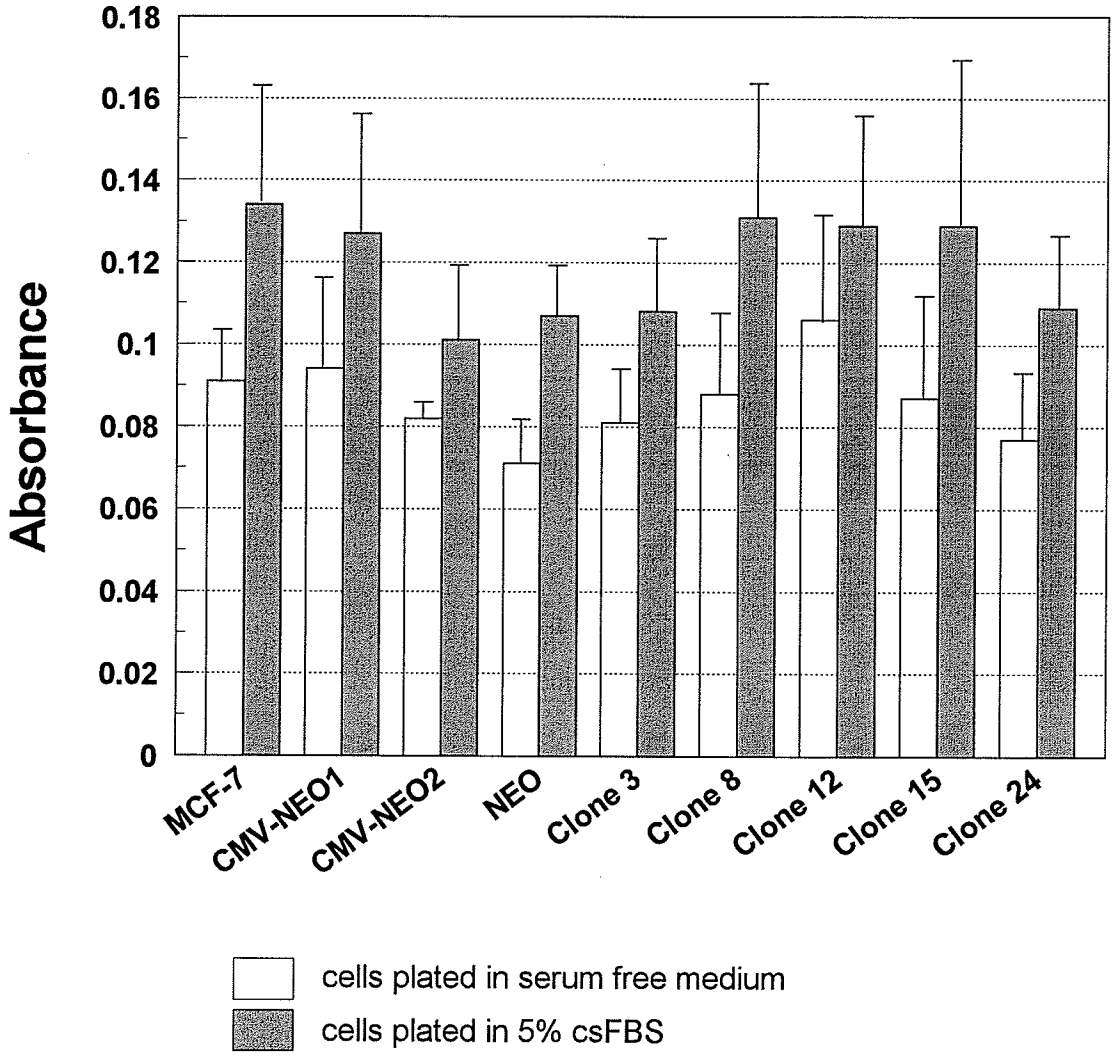

Absorbance
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
MCF-7
CMV-NEO1
CMV-NEO2
NEO
Clone 3
Clone 8
Clone 12
Clone 15
Clone 24
cells plated in serum free medium
cells plated in 5% csFBS

Fig. 9 ⟹

Figure 9. Effect of intracellular IGF-II on MCF-7 cell proliferation in SF medium.

All cells were plated in serum-free medium as described in MATERIALS AND METHODS. The medium was replaced with fresh medium every three days. MTT assay was performed on cells on the days indicated. Points, mean ± SE of triplicate determinations. CMV-IGF-II clones 3, 15, and 24, CMV-NEO2 clone, and NEO clone showed growth characteristics similar to MCF-7 wild-type cells (not plotted).

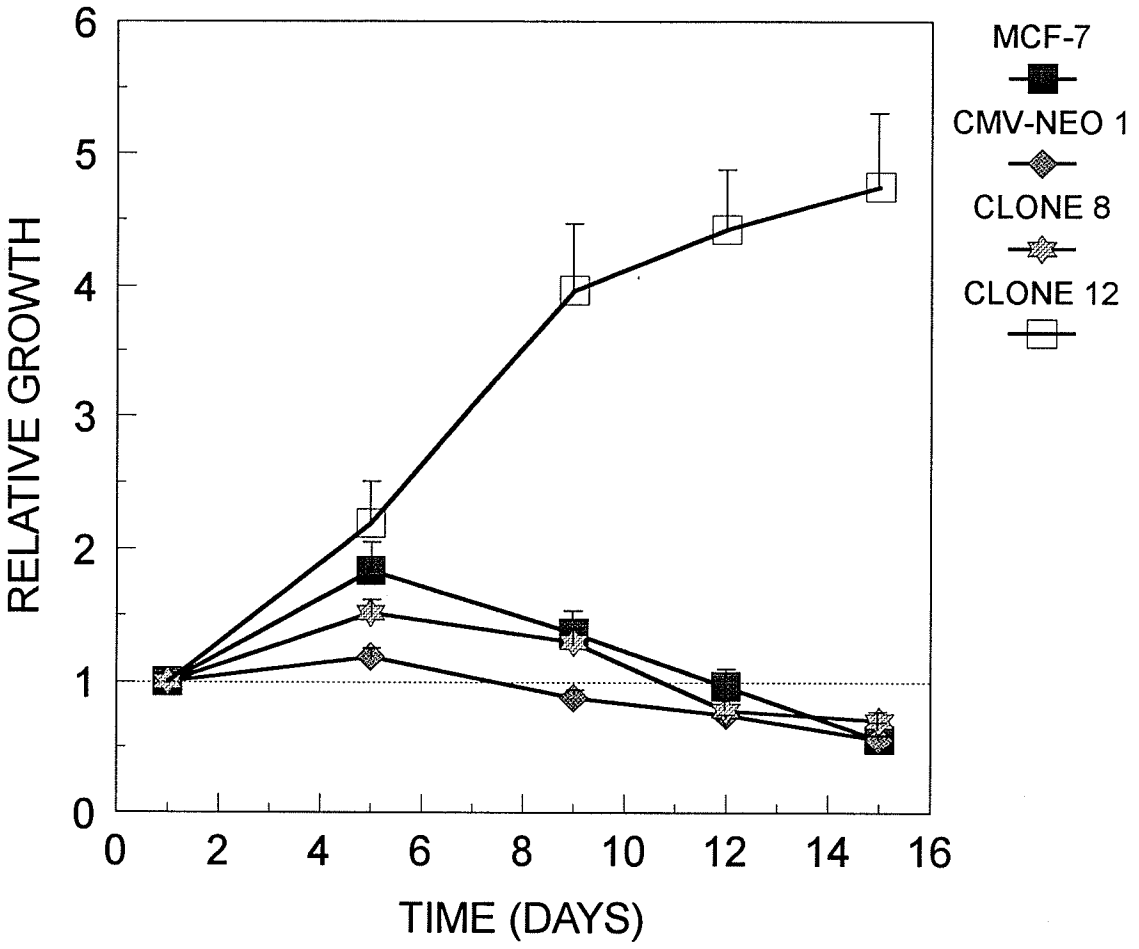
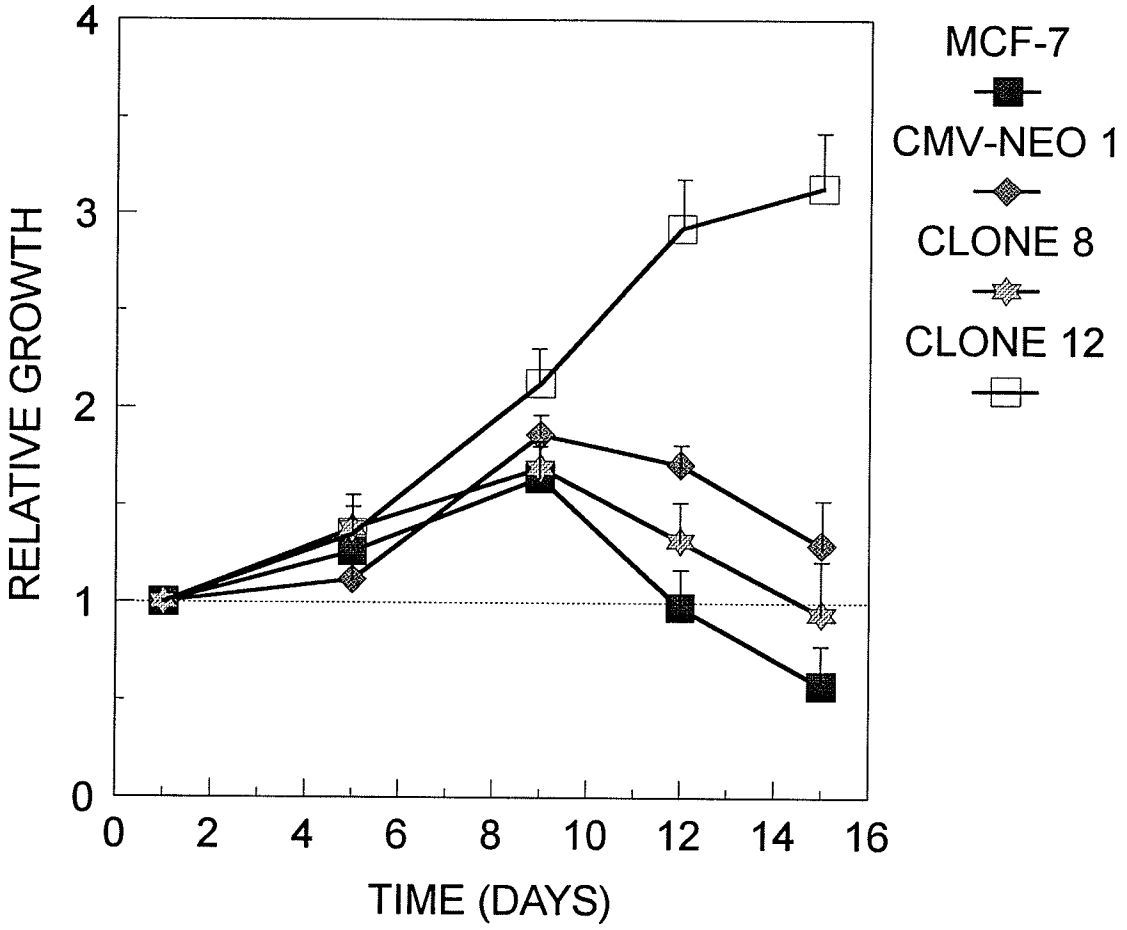

RELATIVE GROWTH
TIME (DAYS)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16
MCF-7
CMV-NEO 1
CLONE 8
CLONE 12

Fig. 10 ⇒

Figure 10. Effect of intracellular IGF-II on MCF-7 cell proliferation in medium containing charcoal-stripped FBS.

All cells were plated in 5%-csFBS as described in MATERIALS AND METHODS. On the following morning, seeding medium was removed, cells were washed and medium was change to serum-free medium. The medium was replaced with fresh medium every three days. MTT assay was performed on cells on the days indicated. Points, mean ± SE of triplicate determinations. CMV-IGF-II clones 3, 15, and 24, CMV-NEO2 clone, and NEO clone showed growth characteristics similar to MCF-7 wild-type cells (not plotted).


RELATIVE GROWTH
TIME (DAYS)
0
1
2
3
4
0
2
4
6
8
10
12
14
16
MCF-7
CMV-NEO 1
CLONE 8
CLONE 12

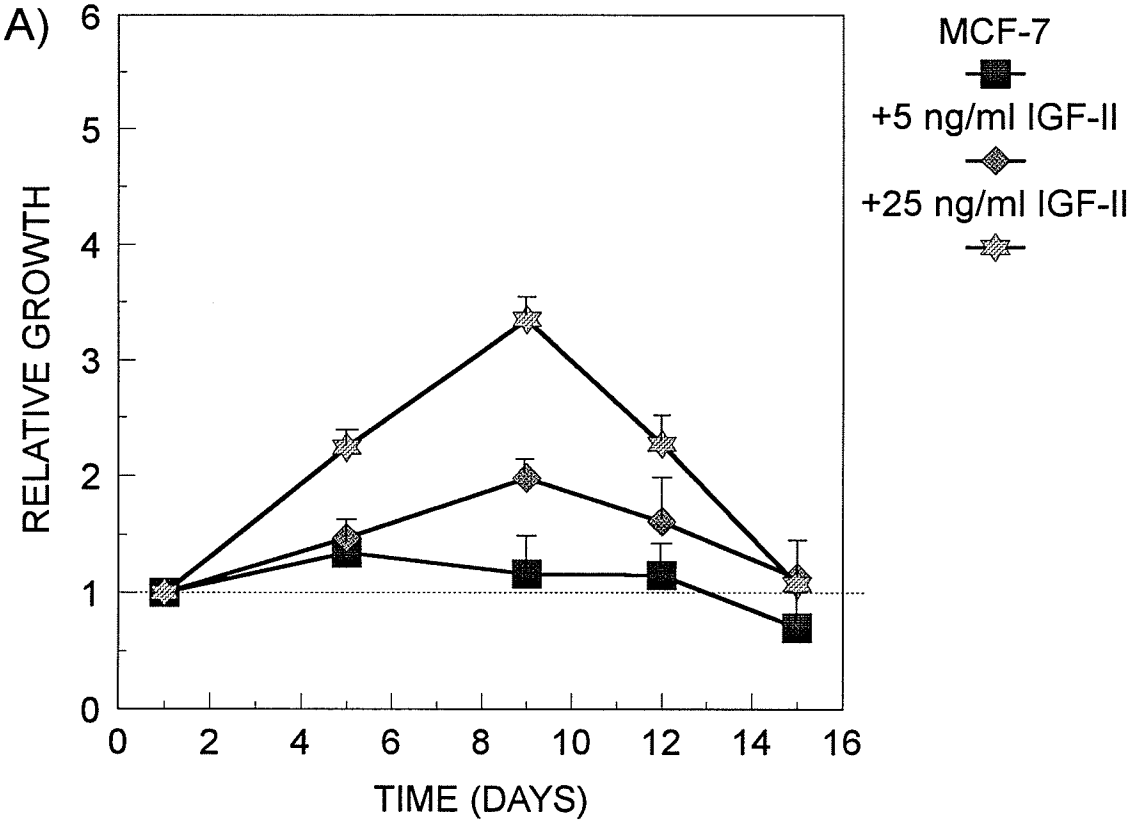
is a necessary property for an appropriate host cell in order to test our intracrine hypothesis. Control cells assayed in SF medium grew very slowly in the absence of any further supplement.

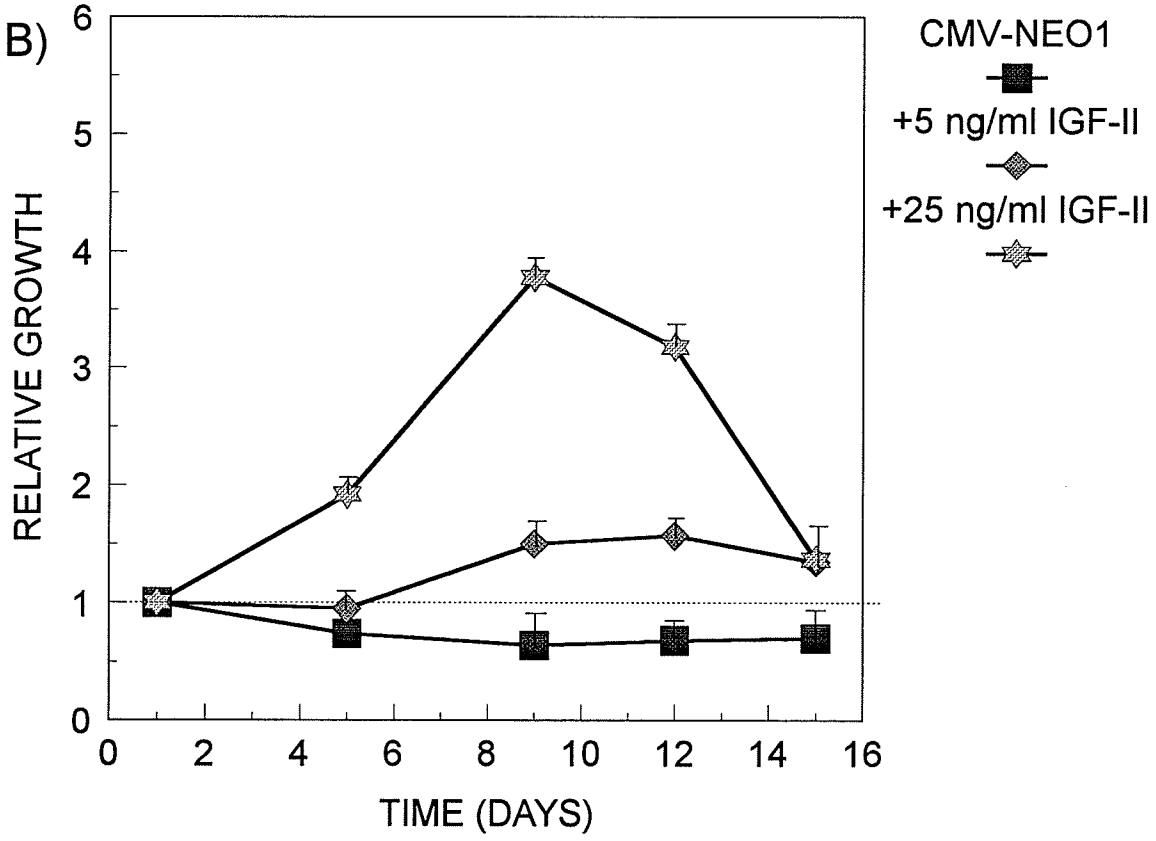
Since the intracellular IGF-II-KDEL expression in MCF-7 cells did not result in intracrine stimulated growth we questioned whether its abundance in the ER was preventing or interfering with the delivery of the type 1 or 2 IGF receptors to the cell surface. Other ligands manipulated to remain in the ER have been shown to bind their newly synthesized membrane binding components, retain them in the ER, and prevent their localization to the cell surface (Rose-John et al, 1993; Buonocore and Rose, 1990). We attempted to indirectly address this question by determining whether our IGF-II-KDEL clones were responsive to exogenous IGF-II. Significant proliferation was found with 5 and 25ng/ml of recombinant IGF-II, which was comparable to that seen in control cells (Figure 11), indicating that the IGF membrane receptors were arriving and functioning at the surface of the cells. Interestingly, despite its rapid proliferation in SF medium, clone 12 was still responsible to exogenous IGF-II.

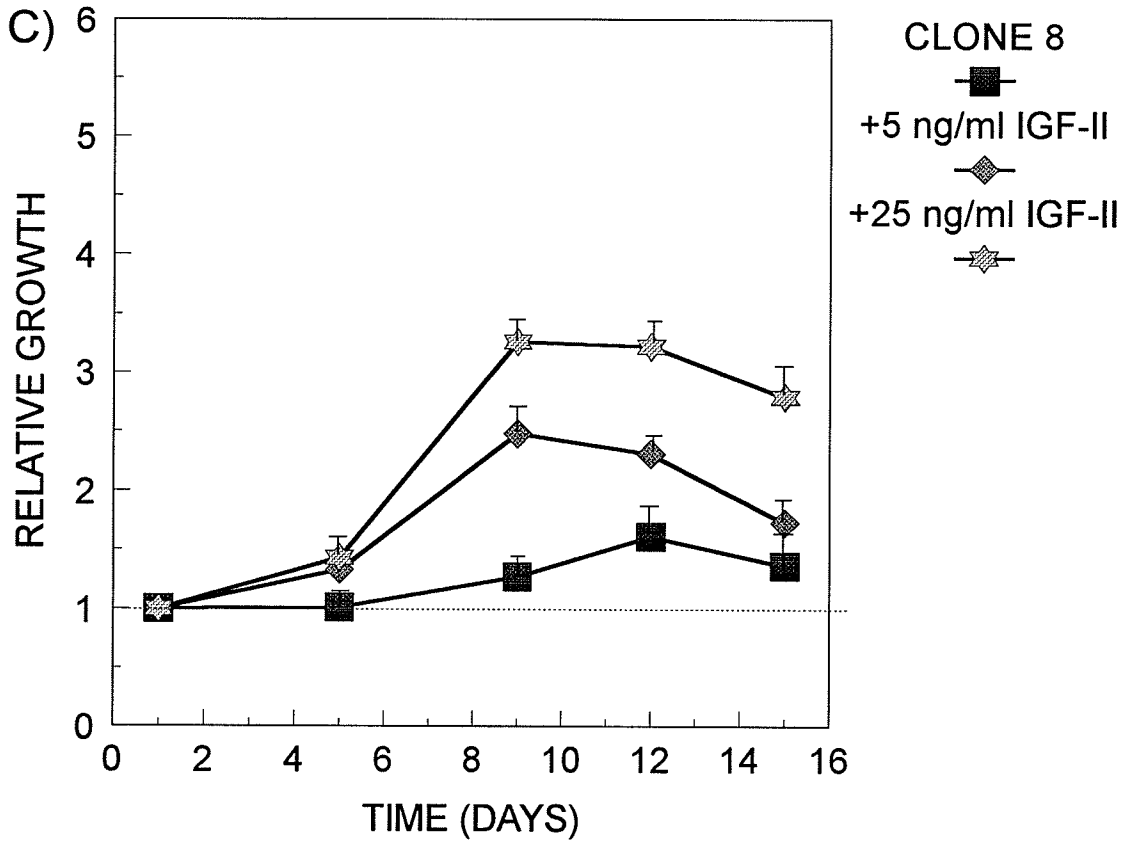
Fig. 11 ⇒

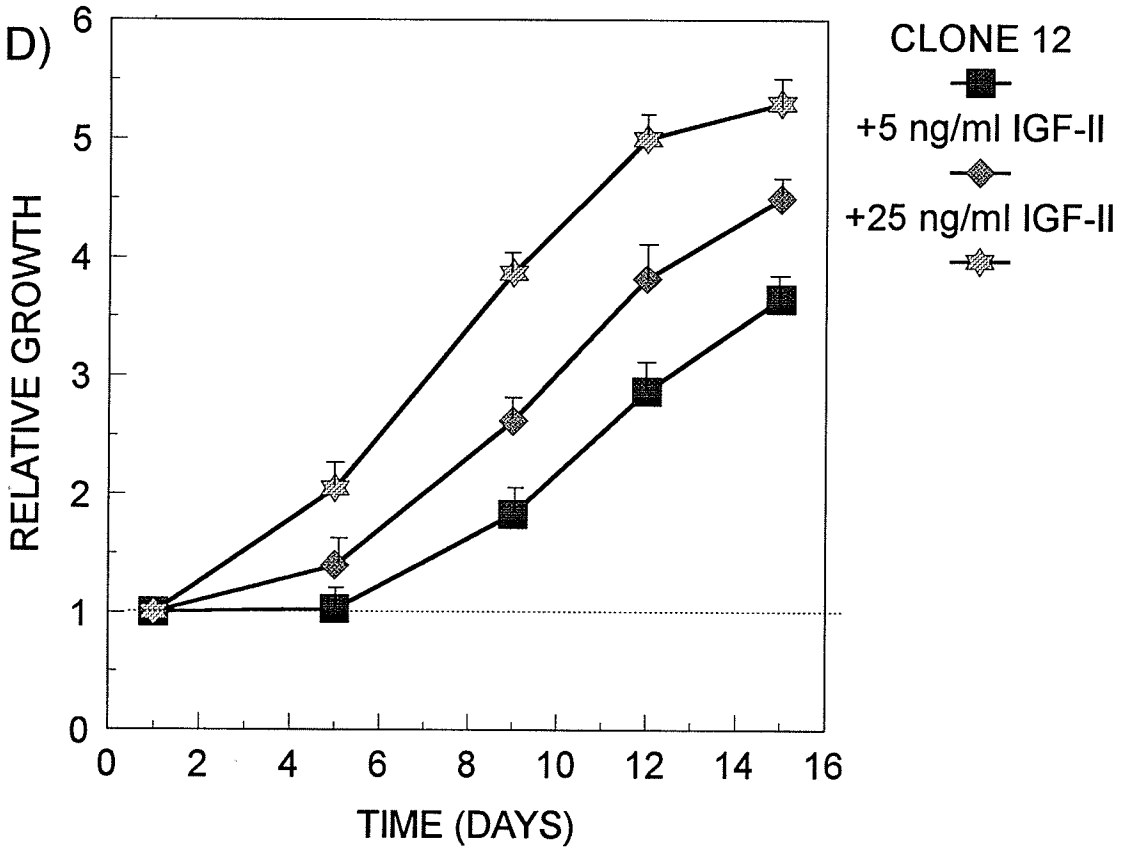
Figure 11. Effect of exogenous IGF-II on MCF-7 wild-type cells and CMV-IGF-II clones.

MCF-7 wild-type cells (A), CMV-NEO1 (B), and CMV-IGF-II clones 8 (C) and 12 (D) were plated in serum-free medium as described in MATERIALS AND METHODS. The medium was replaced with fresh medium every three days. Exogenous recombinant IGF-II was added at 5ng/ml and 25ng/ml, 24 hrs after plating, and every three days when medium was changed. MTT assay was performed on cells on the days indicated. Points, mean ± SE of triplicate determinations.


A)
RELATIVE GROWTH
TIME (DAYS)
MCF-7
+5 ng/ml IGF-II
+25 ng/ml IGF-II


B)
RELATIVE GROWTH
TIME (DAYS)
CMV-NEO1
+5 ng/ml IGF-II
+25 ng/ml IGF-II


C)
RELATIVE GROWTH
TIME (DAYS)
CLONE 8
+5 ng/ml IGF-II
+25 ng/ml IGF-II


D)
RELATIVE GROWTH
TIME (DAYS)
CLONE 12
+5 ng/ml IGF-II
+25 ng/ml IGF-II

DISCUSSION

Polypeptide growth factors are important members of a complex set of regulatory elements involved in normal and neoplastic cellular growth and differentiation. Growth factors mediate their biological response by binding to and activating specific surface receptors which in turn trigger secondary messenger systems. A complete understanding of the growth factor/receptor system may provide relevent therapeutic strategies to inhibit malignant cell growth. IGF-II is an important growth factor for fetal development and is also expressed by neoplastic cells and influences neoplasia from cellular proliferation to malignant transformation and motility (El Badry et al, 1990; Minniti et al, 1992; Stracke et al, 1989). In addition to autocrine and paracrine influences of IGF-II on breast cancer *in vitro* and *in vivo*, experimental observations have been made which could be explained by an intracrine mechanism of IGF-II (Arteaga, 1992; Yee, 1992; Reddy et al, 1992; Dubois et al, 1993).

The KDEL Sequence was Sufficient for Retention of High Levels of IGF-II.

In the present study we isolated MCF-7 clones which synthesize and maintain IGF-II inside the cell. A well defined KDEL retention sequence, found attached to proteins maintained in the lumen of the ER, was added to the COOH terminus of IGF-II. This mechanism of intracellular retention requires a receptor which recognizes and continuously retreives KDEL containing proteins from an early golgi compartment and prevents their secretion. It has been reported that a strong promoter could result in the saturation of the KDEL retention system, resulting in a slight leakage of KDEL-containing proteins into the extracellular medium (Dunbar et al, 1989; Zagouras and Rose, 1989; Rose-John et al, 1993). Despite the use of a strong constitutive promoter (CMV), from which IGF-II-KDEL expression was derived, it appeared that little IGF-II-KDEL was leaked into the medium. Our studies are in agreement with other investigators who successfully maintained high levels of a normally secreted protein inside the cell by this KDEL retention mechanism (Pelham, 1988; Bejcek et al, 1989; Andres et al, 1990; Lewis and

Pelham, 1992b). Similarly, others found that the percentage of IL-6-KDEL which was secreted, whose expression in human hepatoma cells (HepG2) was driven by the metallothionein promoter, was unaltered by cells whether in the absence of $ZnCl_2$ or when stimulated with $ZnCl_2$ to synthesize 10× the amount of IL-6-KDEL protein (Rose-John et al, 1993). These findings appear to rule out the possibility that the saturation of the retention machinery was causing the secretion of IL-6-KDEL.

There are several possibilities to explain these conflicting results concerning the retention of KDEL containing proteins and the saturation of this retention mechanism. Since these proteins are being altered to remain intracellularly, upstream sequences of these normally secreted proteins may be incompatible with correct protein folding and could mask the KDEL sequence. In support of this notion is the finding that complete retention of IL-6-KDEL was achieved when 14 amino acid residues of protein disulfide isomerase, a resident ER protein involved in protein assembly, was added to the COOH terminus of IL-6 followed by the KDEL motif (Rose-John et al, 1993). Alternatively, these upstream sequences may also be involved in the retention of ER proteins. Amino acid sequence analysis identified acidic amino acid residues upstream of the KDEL sequence in some resident ER proteins (Munro and Pelham, 1987; Denecke et al, 1992). The lack of such acidic amino acids may explain why only partial retention of IL-6 and rat growth hormone was achieved by the KDEL sequence (Zagouras and Rose, 1989); whereas, complete retention of IL-6 only occured after the addition of a 14 amino acid extension which contained an acidic EEDDD (Glu-Glu-Asp-Asp-Asp) sequence.

It would appear, however, that acidic amino acids are not essential for complete intracellular retention since many resident ER proteins do not contain such a concentrated acidic cluster. In addition, the T-cell surface protein CD4 (Buonocore and Rose, 1990) and lysozyme (Lewis and Pelham, 1992b) do not contain acidic amino acids near its carboxyl terminus but the KDEL sequence was sufficient for their retention in the ER. Similarly, a few acidic amino acid residues are present near the C-terminus of IGF-II but are not situated in close proximity with each other. Despite this, IGF-II was sufficiently

accumulated intracellularly. Perhaps these additional highly charged upstream sequences may facilitate binding to the KDEL receptor and thus enhance ligand retention at high concentrations.

Proliferation of MCF-7 Cells was not Affected by Intracellular IGF-II-KDEL.

Although IGF-II preferentially binds to the type 2 IGF receptor, the majority of its mitogenic effect is blocked by αIR3, a monoclonal antibody directed against the type 1 IGF receptor (Osborne et al, 1989; Cullen et al, 1990). This antibody, however, had no effect at low IGF-II concentrations in MCF-7 cells (Mathieu et al, 1990), suggesting that the type 2 receptor mediates IGF-II effects at low concentrations in these cells (Cullen et al, 1990). Similarly α-IR3 inhibited most, but not all, of the IGF-II mitogenic effect in MDA-MB-231 ER- cells (Osborne et al, 1989). Thus the type 1 receptor predominantly mediates the growth effect of IGF-II, but the type 2 IGF receptor may have a physiological role in the growth of some cells. Our goal was to determine if IGF-II could stimulate MCF-7 cell growth in intracellular compartments during receptor synthesis or transport, and were not initially concerned whether this proliferation was mediated by the IGF type 1 or type 2 receptor.

Growth factor and growth factor receptor biosynthesis and delivery to the extracellular fluid and the cell surface, respectively, occurs via the same pathway involving the endoplasmic reticulum-Golgi complex. Since both IGF-II and the IGF receptors are synthesized by the same cell, the potential exists that IGF-II interacts with its receptors within various compartments of the secretory pathway (see Figures 3 and 4). Intracellular receptor activation and a growth response have been observed by similar mechanisms in other systems (Huang and Huang, 1988; Bejcek et al, 1989; Logan, 1990; Vignon et al, 1992; Sherman et al, 1993; Taylor et al, 1993). However, no increase in cellular proliferation was observed due to intracellular IGF-II accumulation.

A number of reasons may explain why some growth factors can activate their receptors

either at the cell surface or intracellularly, whereas others only act at the cell surface. The most probable is that ligand-receptor interactions cannot physically occur in intracellular compartments during biosynthesis and intracellular transport due to inappropriate cellular localization or protein structure. Although some receptors and ligands may not be routed via the same compartments on their way to the cell surface (Vlodavsky et al, 1987; Kandel et al, 1991; Mignatti and Rafkin, 1991; Mignatti et al, 1992; Shain et al, 1992), this possibility seems unlikely for the IGF family of receptors and ligands since their transport occurs through the conventional ER-Golgi route. Instead, ligands and receptors in close proximity may not have the correct tertiary conformation required for binding to occur intracellularly. It is not known if the IGF-II-KDEL peptide has assumed correct folding because it lacks the E-domain. In addition, the KDEL extension to the IGF-II peptides C-terminus by site-directed mutagenesis may mask amino acids or protein regions necessary for binding to its receptors. We tend to favour the latter since several high molecular weight and variant forms of biologically active IGF-II have been characterized with E-domain extensions. The presence of these C-terminal extensions did not affect binding to the IGF type 1 (Hammarberg et al, 1991; Perdue et al, 1991) or type 2 (Gowan et al, 1987; Hammarberg et al, 1991) receptors and in some cases were more potent in stimulating growth and differentiation than mature IGF-II (Gowan et al, 1987; Schwartz et al, 1990; Perdue et al, 1991).

Alternatively, the ligand is able to bind to its receptor but activation of secondary messenger systems (signal transduction) only occurs at the cell surface. For example, arrival to the cell surface was found to be a prerequisite for the phosphorylation and activation of the EGF receptor (Van de Vijver, 1991). Studies measuring tyrosine phosphorylation of the EGF receptor found that only the mature 170-kDa protein, which contains both high mannose and complex oligosaccharides, was phosphorylated, whereas the 160-kDa precursor was not (Di Marco et al, 1989; Van de Vijver, 1991). Oligosaccharide processing itself is not essential for EGF receptor activation, rather it is cell surface localization that is necessary. In the case of the IGF receptors, processing in the Golgi complex is not required for activation of the IGF type 1 receptor.

Attempts to identify cellular localization of IGF-II-KDEL by immunocytochemistry was unsuccessful due to the lack of a good antibody available for this procedure. The Amano anti-IGF-II monoclonal antibody we used has been used primarily for radioimmunoassay. Presumably, IGF accumulates in the ER and post-ER salvage intracellular vesicles. We have no reason to assume otherwise since IGF-II accumulates intracellularly due to the COOH-terminal KDEL extension which is bound by a receptor that continuously retreives such proteins leaving the ER and returns them to the ER. Since newly synthesized IGF receptors are routed through the ER, receptor binding would be theoretically possible in the ER and in early post ER compartments.

We found that exogenous IGF-II at physiological concentrations was adequate to significantly increase the growth of MCF-7 clones expressing IGF-II-KDEL. When IL-6-KDEL was expressed in human hepatoma cells (HepG2), its ER retention led to the prevention of surface expression of the IL-6 receptor (Rose-John et al, 1993). As a result, these cells became unresponsive IL-6 and did not bind ^{125}I-IL-6. IL-6-KDEL was, therefore, interacting with its newly synthesized receptor and retaining it in the ER. Similarily, other investigators found that the expression of CD4-KDEL protein inhibited the cell surface expression of the HIV-1 glycoprotein gp120 (Buonocore and Rose, 1990). Our transfected cells were responsive to exogenous IGF-II which is in contrast to the findings of Dai et al (1992) who found that intracellular IGF-IA-KDEL partially blocks receptor delivery to the cell surface and the transduction mechanisms initiated by exogenous IGF-I. They speculate that IGF-IA-KDEL binds its receptor and relays a mild signal intracellularly which result in the desensitization of these cells to exogenous IGF-I. Therefore, it is possible that the exogenous IGF-II could have stmulated the IGF-II-KDEL clones via the type I receptor; this possibility can be tested using the αIR3 anti-type I receptor antibody.

Isolation of an MCF-7 Clone able to Proliferate in the Absence of Serum.

While assaying our IGF-II-KDEL clones for growth, we observed that clone 12 was able

to grow in serum-free medium. This growth could not be attributed to the intracellular retention of IGF-II since several other clones produced even higher levels of IGF-II but displayed growth characteristics which were similar to control cells. Serum-free growth is often the result of over expression of autocrine and/or intracrine growth factors. Analysis by RIA did not not detect IGF-II in medium conditioned for three days indicating that the serum-free growth displayed by clone 12 was not due to the leakage of IGF-II-KDEL into the extracellular medium.

Attempts have been made to determine the factors involved in the self stimulation of cellular growth in the absence of serum. Cell proliferation and the increase in the levels of mRNA of genes necessary for DNA synthesis typically requires at least two growth factors: PDGF and IGF-I (Jaskulski et al, 1988). These same investigators suggest that the production of IGF-I is necessary, and, in some cases, its overexpression alone is sufficient to maintain growth in serum-free medium without any further requirement for exogenous growth factors (Pietrzkowski et al, 1992). We, therefore, looked for IGF-I expression in clone 12. However, preliminary analysis of our clone 12 by RT-PCR did not detect any IGF-I transcript. It is possible that in clone 12 the site of integration of the transfected DNA has resulted in the activated expression of growth-promoting genes. Alternatively, DNA integration may have disrupted cellular genes that are growth suppressors. Either scenario would result in aberrant growth of clone 12.

The progression of breast tumors from hormone dependent to hormone independent growth has impeded treatment by endocrine therapy. The discovery of self-sustaining autocrine growth factors which modulate tumor proliferation and invasion suggest that estrogen and growth factors probably act synergistically and mutually facilitate their actions during the cell cycle. The breast cancer model is now known to consist of numerous interacting factors and diverse cell populations whose successful therapy requires an integration of these multiple parameters. The loss of estrogen dependence for growth is characteristic of phenotypic changes associated with malignant progression. Constitutive expression of growth factors by ER- tumor cells has been suggested to

override their requirement for estrogen. Since clone 12 was isolated from ER+ MCF-7 breast cancer cells, further characterization of this clone may provide useful information about early mechanisms involved in progression from hormone dependence to hormone independence.

Future Considerations.

Growth factors have emerged over the last decade and a half as critical regulators of tumor cell growth. IGF-II has been hypothesized by some investigators to stimulate cell proliferation by an intracrine mechanism, but our studies do not support this hypothesis. MCF-7 clones which maintain IGF-II intracellulary were characterized and intracellular IGF-II was not able to stimulate MCF-7 cell growth. Our IGF-II-KDEL protein product was confirmed, by RIA, to be immunologically reactive. Biological reactivity assay for the IGF-II-KDEL protein was not done due to time restraints. It will be necessary, in the future, to determine the receptor-binding and biological properties of the IGF-II-KDEL.

The isolation of an MCF-7 clone able to grow without any serum may reveal novel genes or mechanisms involved in progression from hormone dependence to hormone independence of breast cancer. Clone 12 may be particularly useful since it was isolated in the presence of estrogen, under normal physiological conditions observed by tumors in nature, rather than selecting cells for their ability to grow in the absence of estrogen. During early stages in the progression of breast tumors towards hormone independent growth, tumor cells are able to proliferate in the absence of estrogen but remain estrogen responsive. Assaying for a mitogenic response to estrogen may reveal the stage of tumor progression our clone is at.

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