Order Number 9119663

Role of insulin-like growth factor II (IGF-II) in 18–54, SF cells

Padhye, Manisha A., Ph.D.

City University of New York, 1991

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

ROLE OF INSULIN-LIKE GROWTH FACTOR II (IGF-II) IN 18-54,SF CELLS.

Manisha A. Padhye

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York.

1991

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

10/27/90
date

Chairman of Examining Committee
Professor James H. Wyche,
Brown University

10/27/90 DEC. 27, 1990
date

Executive Officer
Professor Peter C. Chabora

Dr. Constance Martin, Hunter College

Dr. Rivka Rudner, Hunter College

Dr. Ray H. Gavin, Brookyn College

Dr. Winston A. Anderson, Howard University

Supervisory Committee

The City University of New York

ACKNOWLEDGEMENT

I wish to acknowledge all the people who have made this thesis, and my entire graduate training possible. First, I thank Dr. James Wyche for his continous guidance, patience and support throughout my work, and for making this project possible. I also thank Dr Rivka Rudner for her role as my graduate advisor. She provided constant encouragement, laboratory space, and above all, the much needed moral support that has been invaluable. My friends and colleagues at Hunter deserve a special thanks for all the help they gave me.

Most of all, I thank my husband, Anil, whose patience, understanding and persistence made this project a very enjoyable one.

TABLE OF CONTENTS

FIGURES

TABLES

INTRODUCTION

The study of the origins of hormone-like activity is revealing considerable complexity in endocrine control systems. Conversely, a degree of simplicity is suggested by observations that most hormones belong to a family group. Related chemical sequences among the members point to evolutionary relationships. In some groups, similar biological activities reflect vestiges of common ancestry. In others however, there is complete divergence of biological functions. One family that has retained some common activity is the insulin family of hormones. The insulin family is comprised of insulin, insulin like growth factors I and II (IGF-I and IGF-II), relaxin, and possibly the B subunit of nerve growth factor (Blundell and Humbel 1980). The biological functions of this group of structurally related peptides have diverged, but some common features have been retained.

Insulin and the IGFs are structurally and functionally related polypeptides. IGFs, which were originally called somatomedins, were initially purified from human sera and from media conditioned by cultured cells. The designation "somatomedins" was introduced by Daughaday in 1979 for substances fulfilling the following four criteria:

(i) their concentrations in serum are regulated by growth hormone

(ii) they stimulate incorporation of sulfate into proteoglycan of cartilage

(iii) they have mitogenic activity in fibroblasts,

(iv) they exert insulin-like effects on adipose and muscle tissue.

INSULIN-LIKE GROWTH FACTORS

The isolation of the somatomedins from human plasma and plasma protein fractions, and the determination of their primary amino acid sequences proved to be difficult tasks because of the low concentrations in the starting materials. In 1978, Rinderknecht and Humbel succeeded in isolating two peptides with molecular weights of about 7,500 from Cohn plasma protein fractions, one basic and containing 70 amino acids, and the second slightly acidic, with 67 amino acids. Although clearly separate entities, approximately 70% identity in amino acid sequences suggested derivation from a common evolutionary precursor (Rinderknecht and Humbel, 1978). An approximately 50% structural homology to proinsulin was also observed (Rinderknecht et al., 1976). This relationship, along with in vitro actions somewhat similar to those of insulin, led Humbel and Rinderknecht to name the peptides insulin-like growth factors I and II (IGF-I and IGF-II). It was subsequently shown that the basic somatomedin-C peptide isolated in Van Wyck's laboratory had a sequence identical to that of IGF-I (Klapper et al., 1983),

and that the peptide isolated by Enberg and associates in 1984 from a somatomedin-A preparation was the same as IGF-II. To avoid furthur confusion in nomenclature, it was proposed by workers in the field that the usage of IGF-I and IGF-II be generally adopted when referring to these peptides (Daughaday et al., 1987).

SIMILARITIES BETWEEN INSULIN AND IGFs

IGF-I and IGF-II are thus two closely related peptides, with amino acid sequences considerably homologous to that of proinsulin (Fig 1). The IGFs are mitogenic, and were originally thought to mediate the effects of growth hormone (somatotropin) on skeletal tissue (Van Wyck, 1984). Although this is partially true for the IGF-I, IGF-II appears to be principally involved in fetal growth and development, and before birth may be under the influence of placental lactogen rather than growth hormone (Lund et al., 1986). Both IGF-I and IGF-II are synthesized as precursor molecules which are processed at both amino and carboxyl ends to yield the mature circulating peptides (Bradshaw et al.,1978; Zapf et al., 1981). In common with proinsulin, both are single chain polypeptides with three intra-chain disulfide bridges. Also, domains B, C, and A, corresponding to proinsulin B chain, C peptide and A chain respectively, can be defined. The IGFs, in addition, contain fourth and fifth domains, (D and E), which are not present

in proinsulin (Blundell et al., 1980). Thus, since the C chain of proinsulin is cleaved, limited amino acid sequence homology between insulin and the IGFs is restricted to the B and A domains. In contrast, the C domains of the IGFs remain as parts of the active forms of the molecules (Rinderknecht et al., 1976; Rinderknecht et al., 1978). The structural similarity between proinsulin and IGFs suggested common ancestry of the corresponding genes.

Moreover, the gene for IGF-I was localized to chromosome 12, whereas those of insulin and IGF-II were on chromosome 11 (Brissenden et al., 1984; Tricoli et al 1984). Since chromosome 12 is evolutionarily related to chromosome 11, these comparative gene mapping data suggest a common ancestry for these sites.

IGFs FROM OTHER SPECIES

IGFs have also been obtained from non-human species. IGF-I was isolated from rat serum by Rubin et al., in 1982, and the complete primary structure was deduced from the nucleotide sequence of rat IGF-I gene by Shimatsu and Rotwein (1987). Only three amino acid differences were noted between rat IGF-I and human IGF-I (Bell et al., 1986). In addition to the three amino acid substitutions noted in rat IGF-I, there is one substitution in the D domain of mouse IGF-I (Honegger et al., 1986). IGF-I isolated from adult bovine serum, is identical to human IGF-I. Porcine IGF-I,

deduced from cDNA clones, is also identical to the human peptide (Tavakkol et al., 1988).

Rat IGF-II, originally called multiplication stimulating activity or MSA, was isolated from the conditioned media of the Buffalo rat liver cell line by Marquardt et al. (1981), and the amino acid sequence was determined. Compared with human IGF-II, it has only five conservative substitutions. As was true for the human peptides, some of the rat IGF-II lacked a N-terminal alanine. Whitfield et al. (1984) confirmed the sequence of rat IGF-II proposed by Marquardt et al. (1981) from a cDNA clone isolated from a Buffalo rat liver DNA library. Dull et al. (1984) screened a fetal rat cDNA library and obtained two recombinant clones which included the entire coding region of the rat IGF-II precursor. The deduced peptide sequence from this clone predicted an amino acid sequence identical to that previously reported, except that serine was present instead of glycine at position 33. This reduced the number of amino acid differences between rat and human IGF-II from 5 to 4. Stempien et al. (1986) determined that mouse IGF-II differed from human IGF-II at six positions.

Thus, it is clear that structures are highly conserved among mammalian IGFs. All IGF-I peptides contain 70 amino acids, and all IGF-II peptides 66 or 67. The substitutions are relatively few and mostly conservative in nature, and indicate that there have been strong constraints inhib-

iting evolutionary divergence of these proteins. Based on the sequence similarities of IGFs and insulin, three dimensional models indicate that the three growth factors can have identical hydrophobic cores. Their short connecting peptides are easily accomodated extensions of the A chain-B terminal in both IGF molecules. This might explain observations that some IGF effects are mediated by interaction with insulin receptor (Megyesi et al., 1974).

FUNCTIONS OF INSULIN AND IGFs

Insulin and IGF-I play roles in numerous physiological processes. Insulin is a primary regulator of rapid anabolic responses, including glucose uptake into muscle and fat cells, glycogen synthesis in liver and muscle, and fat synthesis in adipocytes (Rosen, 1987; Kahn, 1985). Its importance is demonstrated in the condition known as diabetes mellitus, in which there are defects in either synthesis of insulin or the ability of cells to respond to the hormone (Ellenberg and Rifkin, 1983). Administration of insulin to hormone deficient individuals can reverse the symptoms.

IGF-I appears to be a prime regulator of growth. (Froesch et al., 1985; Rechler et al., 1985). Although the circulating peptide is produced mainly in the liver in response to growth hormone, growth hormone promotes local-

ized IGF-I synthesis in several target cell types. Although growth hormone may have some direct effect on cells, IGF-I is the major mediator of its effects on somatic growth (Salmon et al., 1957). The role of IGF-I as a primary growth mediator was deduced from the following observations:

(i) infusion of IGF-I into growth hormone deficient rats can restore growth (Schoenle et al., 1982),

(ii) injections of IGF-I directly into the tibial epiphyseal plate can stimulate cartilage proliferation (Isaakson et al., 1985) and

(iii) certain pygmies in Africa produce normal amounts of growth hormone but have decreased concentrations of serum IGF-I (Merimee et al., 1981; Merimee et al., 1989).

The physiological role of IGF-II in adults is not known. As mentioned earlier, evolutionary conservation is such that human and rat IGF-II share 95% amino acid sequence homology (Froesch et al., 1985). Although a specific in vivo physiologic role for IGF-II has yet to be defined, IGF-II stimulates DNA and protein synthesis in vitro , and may function as a fetal growth factor (Adams et al., 1983). Growth hormone deficiency causes a partial decrease in the plasma concentrations of IGF-II (Rechler et al., 1985). However, in pygmies with low IGF-I, the plasma concentration of IGF-II is near normal (Merimee et al., 1981). Thus, IGF-II is not sufficient to stimulate post natal growth. Also, nude mice with IGF-II producing tumours, and conse-

quently elevated IGF-II, do not show an increased growth rate (Wilson et al., 1987). In rats, plasma levels of IGF-II decrease dramatically after birth, suggesting that IGF-II may, again, play a pivotal role in fetal growth and development (Adams et al., 1983). Rat IGF-II was detected in high levels in fetal serum and in supernatants of fetal liver explants (Reeve et al., 1985) and embryo fibroblasts (Scott et al., 1985). IGF-II levels in the rat have been shown to be 20-100 fold higher in fetal serum than in maternal serum, and they decline a few days after birth (Ashton et al., 1985). IGF-II-like molecules are secreted by the extra-embryonic yolk sac mesoderm, suggesting that IGF-II is expressed in a mesodermal lineage-specific manner (Moses et al., 1980).

In humans, however, plasma levels of IGF-II actually increase after birth, supporting the view that in humans IGF-II functions are not limited to fetal development. The messenger RNA (mRNA) for IGF-II is present in many cell types (Ashton et al., 1985). IGF-II mRNA is also elevated in several kinds of tumor cells, suggesting that IGF-II could act as a paracrine or autocrine growth factor. Emerging evidence suggests that insulin and IGF-II have neurotrophic properties (Mill et el., 1985). Neurite formation is stimulated by physiological concentrations of IGF-II in sensory, sympathetic and neuroblastoma cells. IGF-II gene is expressed in fetal rat brain (Lund et al., 1986; Graham

et al., 1986), and binding sites for IGF-II are present in brain homogenates. This supports the postulation that IGF-II plays an important role in central as well as peripheral nervous system.

IGF-like peptides are also secreted by various tumor tissues, and by transformed and untransformed cultured cell lines (Reeve et al., 1985; Scott et al., 1985). IGFs may thus play important roles in deregulation of growth control in certain types of tumors, again through autocrine or paracrine mechanisms.

Action

To mediate the various effects of a peptide, the first and common essential step is interaction with specific, high affinity, cell surface receptors. Each of the three polypeptides has its own distinct receptors. The ligands, in general, can bind to each other's receptors with varying affinities. Table I lists the comparisons between the IGF and insulin receptors. The presence of all three receptors in most cells has complicated the assignment of a particular response to a specific receptor type.

Ullrich et al., in 1985 determined the complete primary structure of the human IGF-I receptor from cloned cDNA. As anticipated, extensive similarity with the insulin receptor was observed, including overall structure, subunit

size, and primary sequence. In common with the insulin receptor, the IGF-I receptor has been found to be a hetero-tetramer composed of two alpha subunits (M_r 135,000) and two beta subunits (M_r 90,000), linked in both cases by interchain disulfide bonds (Kasuga et al., 1982; Jacobs et al., 1983). Both subunits are glycosylated, and both are exposed to the external surface of the cell. The alpha subunits contain the binding sites for insulin or IGF-I, whereas the beta subunits contain a transmembrane domain, an ATP binding site, and a tyrosine autophosphorylation site (Rubin et al., 1983; Zick et al., 1984). These findings are consistent with the observation that insulin and IGF-I can induce phosphorylation of tyrosine residues on the beta subunits of their own receptors, as well as on exogenous protein substrates. However, while the insulin receptor has been localized on chromosome 19 (Sara et al., 1982), the IGF-I receptor has been mapped to the distal band of the long arm of chromosome 15 (Ullrich et al., 1986). These differences suggest that the insulin and IGF-I receptors are not only the products of distinct genes, but are probably subject to different forms of regulation. Jacobs et al. (1986) have demonstrated similar maturation processing of the insulin and IGF-I receptors, although the respective precursors could be specifically immunoprecipitated by anti-insulin or anti-IGF-I receptor antibodies, confirming that the precursors for the two receptor types are distinct polypeptides.

Studies performed with IGF-II have indicated the existence of a receptor (Type II) which is structurally and immunologicaly distinct from insulin and IGF-I receptors. Following affinity crosslinking and SDS-PAGE, this receptor migrates with an M_r of 220,000 in the unreduced state, and 250,000 following reduction, suggesting that it is a single chain polypeptide with internal disulfide bridges which act to compact the molecule (Kasuga et al., 1981; Massague and Czech 1982). Binding of 125-I-IGF-II to this receptor is preferentially inhibited by IGF-II as compared with IGF-I, with no affinity for insulin. Insulin appears to activate the Type II receptors on the cell surface (Oppenheimer et al., 1983). The IGF-II receptor does not bind insulin, but it does bind IGF-I with low affinity. Although phosphorylation of the type II receptor has been observed, this action could be mediated by the Type I receptor (Hascall et al., 1985). There is still no definitive evidence for intrinsic tyrosine kinase activity, and a study by Corvera et al. (1986) demonstrated that the IGF-II receptor lacks a tyrosine kinase sequence.

Studies of the IGF-II receptor indicate that the apparent molecular mass of this receptor in the absence of N-glycosylation is 232,000, and that glycosylation is required for acquisition of binding activity (MacDonald and Czech, 1985). The receptor is synthesized initially as a 245,000 M_r precursor having 4-6 high-mannose oligosaccha-

ride side chains. Mannose removal and terminal sialylation convert this precursor to the 250,000 M functional receptor. Amino acid sequences deduced from rat cDNA clones encoding IGF-II receptor closely resemble those of the bovine cation-independant mannose-6-phosphate (Man-6-P) receptor, suggesting they are identical structures (Lobel et al., 1987). It has also been shown that the IGF-II receptor contains cooperative, high affinity binding sites for both IGF-II and the mannose-6-P containing protein (Jonas et al., 1985; Misra et al., 1986). Since the two cDNAs were isolated from different species (human and bovine for the IGF-II and mannose-6-P receptors, respectively), this degree of sequence identity is consistent with a single gene encoding both proteins. Additional support for this hypothesis comes from the finding that mRNA synthesized from the isolated cDNA could be used by frog oocytes to produce a protein that binds IGF-II and is recognized by antibodies to the Man-6-P receptor (Morgan et al., 1987). More recently, purified human (Roth et al., 1987) and rat (MacDonald et al., 1988) IGF-II receptors have been found to interact with antibodies to the Man-6-P receptor, and with Man-6-P. Also, the purified Man-6-P receptor was found to bind IGF-II with the same high affinity as IGF-II binds to its own receptor (K_D = 0.2 nM)(Tong et al., 1988) and a stoichiometry of one IGF-II molecule per molecule of Man-6-P receptor. Finally, the amino acid sequence of the human Man-6-P receptor was found to be 99.4% identical with the sequence

of human IGF-II receptor (Oshima et al., 1988).

The amino acid sequence of the IGF-II receptor predicts a structure with a single transmembrane domain comprising 93% of the receptor molecule, and a relatively small cytoplasmic domain of M_r 18,000 (Morgan et al., 1987). Neither the extracellular nor the intracellular domains of the IGF-II receptor shares homology with the insulin or IGF-I receptor. Thus, one might think that such a receptor would be unlikely to mediate responses similar to those of IGF-I and insulin receptors. However, the receptor for nerve growth factor also has a small cytoplasmic domain with no apparent signal transducing abilities (Radeke et al., 1987), yet this receptor can mediate some of the same responses as the insulin receptor (Mill et al., 1985).

CROSS-REACTIVITIES OF INSULIN AND IGFs

It was observed that to elicit growth, the concentration of insulin required was very high or supraphysiological (1 ug/ml, or higher) for most cell lines. H-35 rat hepatoma cells are unusual, in that they respond to physiologial levels of insulin (Koontz et al., 1975). At such high concentrations of insulin, it is unlikely that biological effects result from furthur occupancy of already satu rated high affinity insulin receptors. Rather, evidence was presented (Rechler et al., 1976) that in human skin fibroblasts, insulin exerts its growth effect by interaction

with low affinity receptors for other hormones, most notably the IGF-I receptors.

The hypothesis that insulin mediates its growth stimulation through IGF receptors was based on binding studies which indicated that insulin competed for ^{125}I-IGF-I binding to chick embryo fibroblasts (King et al., 1986; Straus, 1981). It was furthur observed that insulin and MSA (rat IGF-II) promote fibroblast growth, and that this effect was non-additive (Rechler et al., 1977). The most positive evidence for insulin mediation of growth via interaction with IGF receptors was obtained in human foreskin fibroblasts (King et al., 1980), in which the high affinity insulin receptor was blocked by an anti-receptor antibody. This did not prevent the stimulation of growth in the cells. In contrast to the results mentioned above, the growth stimulatory effects of insulin on some cell lines such as rat liver cells, appear to be mediated by binding to insulin receptors (Flier et al., 1977). This is proven by the fact that in rat hepatoma cells, a very low dose of insulin is sufficient to elicit a growth response. These cells have IGF Type II but no IGF Type I binding sites. Based on these data, it is proposed that both IGF-I and insulin can participate in the regulation of cell growth and metabolism *in vivo*, and that their roles may be defined, in part, by the resident receptor populations of the various tissues. In general, the insulin receptor has been implicated in more

rapid anabolic responses, whereas the IGF-I receptor is more likely to mediate proliferation (King et al., 1980). However, insulin acts through its own receptor to stimulate proliferation in certain cell types (Straus, 1984), and IGF-I can mediate rapid anabolic responses in others (Heaton et al., 1984; Yu et al., 1984).

Attempts to determine which responses are mediated via IGF-II receptor have been less successful. Some investigators have assigned particular responses to the IGF-II receptor on the basis of correlations between data of binding studies and activity curves (Beguinot et al., 1985). However, several studies in which antibodies were used to block responses through the three different receptors led to the conclusion that the IGF-II responses examined were mediated via either IGF-I or insulin receptors (Mottola et al., 1984; Conover et al., 1986). It has even been proposed that the IGF-II receptor does not play a role in transmembrane signaling (Mottola et al., 1984).

In the last few years however, evidence from several systems has suggested that IGF-II can stimulate a response through its own receptor. The responses that are linked include stimulation of calcium ion influx in 3T3 fibroblasts (Nishimoto et al., 1987), amino acid uptake in human myoblasts (Shimizu et al., 1986), DNA synthesis in a human erythroleukemia cell line and a rat cell line (Tally et al., 1987), and glycogen synthesis in hepatoma cells

(Hari et al., 1987). In both of the latter studies, the responses to IGF-II were not completely blocked by specific antibodies to insulin and IGF-I receptors. In one study, an antibody to IGF-II was even found to stimulate a specific biological response, activation of glycogen synthesis (Hari et al., 1987).

However, discriminating the functional roles of the receptors of the three ligands has not been easy because of the overlapping affinities of insulin and the IGFs for each other's receptors, and because of the use of incompletely purified IGF preparations in the binding and biological studies. Also, possible heterogeneity in binding properties of these receptors in different tissues and species, adds additional complexities.

PURPOSE OF THESE STUDIES.

18-54, SF is a rat cell line of neuroendocrine origin which can be grown in tissue culture with serum free chemically defined media. The fact that these cells grow in serum free media offers the advantage of studying a well differentiated cell line that enables investigation of the effects of exogenously added substances on cell growth, growth factor regulation and gene expression. The physiological and molecular mechanisms by which 18-54,SF cell growth can be continously maintained in serum free medium are not

known. Neither epidermal growth factor (EGF), nor transforming growth factor (TGF) is synthesized by these cells (Lieberman and Wyche, unpublished data). These cells do, however, synthesize and secrete IGF-II (Wilson et al., 1987).

Studies on how growth is regulated in this cell line can provide insights into more general aspects of cellular growth and proliferation control.

It was postulated that cellular growth and differentiation in 18-54, SF cells is regulated by IGF-II. This has led to exploration of the roles of the IGF-II, insulin, and IGF receptors, and of IGF-II gene expression that may operate in this system. Since 18-54, SF cells synthesize IGF-II mRNA but not IGF-I mRNA (Bell and Wyche, unpublished data), the study focused on the understanding of the cell and molecular biology of IGF-II in 18-54, SF cells.

Since insulin and IGF-I have been implicated as important regulators in cellular proliferation, the effects of insulin and insulin-like growth factors on the cell number of 18-54, SF cells were investigated. It was found that neither insulin nor IGF-I caused any increase in cell growth. However, IGF-II significantly increased cell numbers in defined media.

It was demonstrated that the 18-54 ,SF cell line has receptors for insulin, IGF-I and IGF-II. Although the insulin and the IGF-I receptors have been shown to invoke a wide variety of anabolic and mitogenic actions, as mentioned above, several reports have failed to support a role for the IGF-II receptor in causing the diverse actions of IGF-II. Mottola and Czech, in 1984, have elegantly demonstrated that the Type II receptor in H-35 hepatoma cells does not mediate IGF-II stimulated DNA synthesis. Rather, the mitogenic effect of IGF-II on this cell line appears to be mediated via the insulin receptor. Similarly, Conover et al., in 1986 have shown that the mitogenic actions of IGF-II in human fibroblast monolayers can be inhibited by a monoclonal antibody directed against the Type-I receptor, despite persistent binding of IGF-II to its own receptor. It was shown in this study that in this cell line the IGF-II actions are mediated through the IGF-II receptor.

The IGF-II gene was isolated by screening a genomic library from the 18-54, SF cells with a rat cDNA probe, and a comparative restriction map was constructed. It was shown to be identical to the map of the IGF-II gene isolated from rat by other studies (Frunzio et al ., 1986; Soares et al ., 1986). In addition, since a full length DNA clone is now available, DNA sequencing can provide some insights into IGF-II gene expression and regulation in 18-54,SF cells.

SPECIFIC AIMS OF THIS STUDY

(i) To determine which polypeptides/growth factors known to be mitogenic in other cell lines can promote increase in cell proliferation in the 18-54, SF cells.

(ii) To determine the amounts of IGF-II secreted in the medium. Furthurmore to check whether the increasing concentrations parallel increases in cell numbers.

(iii) To determine if there are specific receptors for insulin, IGF-I and IGF-II on the 18-54,SF cells.

(iv) To use a polyclonal antibody against the IGF-II receptor, and to determine whether the mitogenic effects of IGF-II can be blocked with a polyclonal antibody directed against the IGF-II receptor. This would enable identification of the receptor type that mediates IGF-II stimulated cell proliferation, since the hormone binds to insulin, IGF-I and IGF-II receptor types.

(v) To make a genomic library, and isolate the IGF-II gene using a rat cDNA probe. The gene can furthur be mapped to study if it is different from that of the previously studied rat IGF-II gene. These studies

would also elucidate the mechanism of regulation of this gene. The 5' end of the IGF-II gene can be studied for the presence of promotors as has been observed by others (Frunzio et al.,1988) in the rat liver IGF-II gene. The role of the promotors and their actions can also be studied to determine how this gene controls growth in the 18-54, SF cells.

MATERIAL AND METHODS

MATERIALS

Coon's Modified Ham's F-12 medium (Coon's medium) was purchased from KC Biologicals (Laxena, KS). HEPES, insulins (bovine, ovine, equine, bovine chains A and B), were obtained from Sigma Chemical Co., St.Louis, MO, trypsin from American Biochemicals Co., Cleveland, OH, and collagen from Worthington Biochemicals, Freehold, NJ. Human monocomponent insulin was purchased from Novo Research, Inc. (Copenhagen, Denmark) and porcine insulin was a generous gift from Eli Lilly, Co., Indianapolis, IN. Epidermal growth factor (EGF), MSA (rIGF-II) and 7S nerve growth factor (NGF) were obtained from Collaborative Research, Inc., Waltham, MA. Pituitary derived fibroblast growth factor (FGF) was purchased from Biomedical Technology Inc., Cambridge, MA.. Pure (Thr-59)-IGF-I was purchased from Amgen Biologicals (Thousand Oaks, CA). Synthetic human IGF-II was provided by Dr. C.H. Li (San Francisco, CA). Prelimnary investigation showed that the natural and synthetic IGF-II preparations were identical in their ability to displace ^{125}I-IGF-II from membrane receptors. rIGF-II purified to homogeneity from media conditioned by 18-54, SF cells was provided by Dr. K. Nishikawa (Kanazawa Medical University, Japan) (Tanaka et al., 1985). This peptide has been totally sequenced, and found to be identical to rIGF-II purified from BRL-3A condi-

tioned medium (Nissley P. et al., 1977); it deviates from hIGF-II at amino acid position 22 (Ser), 32 (Ser), 35 (Ala), and 36 (Asn).

Restriction enzymes were purchased from New England Biolabs, or from Bethesda Research Laboratories. Nitrocellulose membranes were obtained from Scheicher & Schuell. ^{35}S-dATP was bought from New England Nuclear.

IODINATION

^{125}I-porcine insulin was obtained from New England Nuclear, Inc., Boston , MA, with batch specific activities ranging from 81-94 uCi/ug. Peptides were iodinated by a modification of the chloramine-T method to specific activities of 150-250 uCi/ug.

5ug of peptide was added to 20ul of 0.3 M potassium phosphate pH 7.4 and 1.5-2 mCi of Na-125-I. Freshly made Chloramine-T (120 ug/ml) was then added to the solution. The reaction was stopped with 0.3 M phosphate buffer containing BSA, a point experimentally determined by maximal incorporation of radioactivity into the trichloroacetic acid precipitate . This usually happened after two additions of Chloramine-T. The reaction mixture was chromatographed on a Sephadex G-50 (150 X 1 cm) column which had been equilibrated with 1 M acetic acid containing 1 mg/ml BSA at 4 ^{o}C. 1 ml fractions were collected and counted on a gamma counter. The

labeled peptide peak could be clearly resolved from ^{125}I-BSA and free iodine.

CELLS

The 18-54, SF cells were initially said to have been isolated from a human pituitary adenoma of an acromegalic female (Wyche and Noteboom 1979). However, recent data from several sources indicate that the cells are of rat, and not human origin. These data include chromosome analysis (Henderson and Nishikawa, personal communication), peptide sequence analysis of IGF-II secreted by 18-54, SF cells (Nishikawa, personal communication), Southern blotting with a cDNA probe for the hIGF-I receptor (Rosenfeld, personal communication), and IGF-II DNA restriction analysis and DNA sequencing (Majumdar, Padhye and Wyche, manuscript in preparation). The human IGF-I receptor monoclonal antibody a-IR-3 (Jacobs et al., 1983), which reacts with human but not rat IGF-I receptors. a-IR-3, in several experiments does not block 125-I-IGF-I binding to 18-54, SF cell membranes (Wyche, unpublished data). Thus, the origins of these cells clearly indicate that they are rat derived. Pathological and histological studies confirm that the 18-54, SF cells are of neuroendocrine origin (Wilson et al., 1987).

Cell Growth

The stock 18-54,SF cells are routinely cultured in serum free Coon's medium (SFC) in 75 cm plastic culture flasks , or in 100 X 20mm dishes with an inoculum of cells, and incubated at 37°C, in a humidified atmosphere of 5% CO_2 :95% air. Although these cells are capable of growth under serum free conditions in SFC, they were sometimes grown in the above medium supplemented with 0.5% fetal bovine serum (Irvine Scientific, CA).

Cell Harvest For Cell Membrane Preparations

Cells used for receptor assays were handled as described above, except that after 24 hours the growth media were removed and fresh SFC added, with and without other ad ditions. The cultures were used 1-2 days after reaching confluency. The procedure has been reported (Lee et al., 1986) and the modification is described below.

To harvest cells for membrane studies, cells were detached from the dishes by incubation in calcium and magnesium deficient PBS supplemented with 2mM EDTA at 37 °C for 10-15 min. The detached cells were removed by pipetting with a solution of SFC, and then centrifuged at 800 X g for 5 minutes. The cell pellet was washed once in SFC and then resuspended in ME buffer, pH 8 (50mM HEPES, 10mM dextrose, 50mM Tris, 125mM $NaCl_2$, 2.5mM CaCl ,5mM KCl and 0.1% BSA).

Cell viability was determined by the trypan blue exclusion method (Philips et al 1973), and found to be 90% or better. Cell number was determined by hemocytometer counting. The cells were resuspended in 5-7ml of buffer and lysed by homogenization for 30 seconds at a setting of 55 on a Tekmar Ultra-Turrax homogenizer. The homogenate was centrifuged in a Sorvall RC-2B centrifuge for 20 minutes at 20,000 X g. The resultant pellet was washed with ME buffer, and resuspended in 3 ml ice cold buffer for each of the 10 dishes (100mm x 20mm) of cells. This gives a protein concentration of 0.2 and 0.5 mg per 100ul. Protein values were determined by the method of Lowry et al. (1951).

Hormone Production and Assays

To assess IGF-II production by 18-54,SF cells, 2 X 10^5 cells were seeded in a 3ml Coon's Modified Ham's F-12 medium containing 0.5% fetal calf serum in 28cm plastic plates. The media were not changed during the 10 day course of the experiment. Three plates were harvested each day. Conditioned medium was aspirated from each plate and centrifuged to remove cellular debris. The supernatant was frozen for subsequent determination of IGF-II concentration. Media samples were chromatographed on a 0.9 X 100 cm column containing Sephadex G-50 (fine), using 0.25M formic acid to separate the somatomedin peptides from any binding proteins. rIGF-II was assayed by rat placental membrane radioreceptor

assay as described below. IGF-I content was determined by a specific RIA that utilized antiserum AS-6 kindly provided by Drs. L.E. Underwood and J.J.Van Wyck (University of North Carolina, Chapel Hill, NC), and distributed for research use through the National Hormone and Pituitary Program of the National Institutes of Health. When the medium was aspirated for IGF assay, cells were detached from the plates using a trypsin-EDTA-collagenase mixture. Following centrifugation, the cell pellet was frozen in 2M NaCl buffer, pH 7.4, containing 40mM Na_2HPO, 10mM NaH_2PO , and 2mM EDTA. The DNA content was determined by the flourescent dye method as described below.

RADIORECEPTOR ASSAY

Radioreceptor assays was performed on three day old rat placenta (Daughaday et al., 1981). Rat placental membrane (120 ug) was added to each 1.5 ml microfuge tube in a total volume of 0.5 ml 0.1 M Tris buffer pH 7.4, containing 0.25 M NaCl and 3% BSA (wt/vol). The reaction mixture included ^{125}I-IGF-II (20,000 cpm representing about 0.4ng of the peptide), as well as conditioned medium which included the IGF-II whose concentration was to be determined. After 16 hours, at 4oC, the tubes were centrifuged at 10,000 X g in a microfuge for 10 minutes. The supernatant was removed by aspiration, and the pellet counted. Control tubes were run in the absence of membrane. IGF-II has been shown to

displace more than 95% of bound ^{125}I-IGF-II, with 5-8 times the amount of IGF-II required for 50% displacement of original binding from rat placental membranes. This shows that nonspecific binding is very limited. The results were then compared to standard curves prepared with up to 100 ng/ml IGF-II.

Flourescent Dye Method

DNA isolated fom the 18-54, SF cells was prepared in phospho-saline buffer, pH 7.4 (0.05 M $NaPO_4$, 2.0 M NaCl). DNA determinations were performed on aliquots containing 1 ug of H 33258 per ml. Hypochromicity of DNA was determined by measuring the increase in absorbance at 260 nm after denaturation.

Receptor Antibodies

The polyclonal antibody R-II-PAB1, which is specific for the IGF-II receptor, was prepared in the laboratory of Dr. Ron Rosenfeld, Stanford University by precipitation of antiserum in 45% saturated ammonium sulfate and resuspension in PBS (Rosenfeld et al., 1986). Monoclonal antibodies A2-1, A1-1, A3-1 and B4-5, which are directed against the rat IGF-II receptor, were obtained following immunization of the BALB/c mice with rIGF-II receptors over agarose immobilized rat IGF-II. These antibodies have been shown to immunoprecipitate the Type-II receptor, but they do not inhibit the

binding of ^{125}I-IGF-II. Monoclonal antibodies were employed in the form of mouse ascites fluid clarified by centrifugation.

Cell Replication Studies

Approximately 4.5 X 10^4 18-54, SF cells were plated in 60 mm X 10 mm dishes in 5 ml of serum free media. Triplicate dishes were supplemented with either PBS or graded concentrations of (Thr-59)-IGF-I, insulin or IGF-II. The cells were incubated for 5 days, detached by trypsin EDTA-collagenase, and counted by a Particle Data Cell Counter and hemocytometer.

To examine the effect of anti-receptor antibody on 18-54, SF cell proliferation, two different methodologies were employed. In the "dense cell" experiment, 5 X 10^4 18-54, SF cells per 60 mm X 10 mm dish were incubated for 5 days in serum free media in the presence of increasing concentrations of r-II-PAB1. At the end of 5 days, cells were detached with trypsin EDTA-collagenase, and counted by a cell counter and hemocytometer.

In the "sparse cell" experiment, approximately 100 cells of 18-54, SF were plated in 4 cm diameter plastic wells (Costar) as single cells, in F-12 medium supplemented with 0.5% fetal bovine serum. r-II-PAB1 was added at the indicated concentrations, either at the time of plating or

24 hours after plating. After 7 days, unless otherwise specified, cell colonies were stained with 0.5% methylene blue in 70% isopropanol. The numbers of colonies were counted, and the number of cells per colony determined by counting under an inverted microscope.

Binding Studies

Binding was assessed with cell membranes in suspension in a total assay volume of 250 ul. 500 ul microcentrifuge tubes were used. To a 200 ul cell suspension (2 X 10^6 cells/ml = 100 ug protein) was added 25 ul of labelled ligand at a final concentration of 0.45 ng/ml, or as stated elsewhere, and 25ul of buffer with and without various concentrations of unlabeled competitor (up to 50 ng/ml). Cells were incubated at 24 ^oC for 60 minutes, or as otherwise stated. The pH, time and temperature conditions were determined to be optimal in preliminary assays. Non-specific binding was determined by measuring the amount of cell bound 125-I ligand in the presence of various concentrations of unlabeled ligand. This was subtracted from the amount bound with labeled ligand alone, i.e total binding. The assay was terminated by centrifugation at 13,700 X g for 3 minutes in a Fisher model 340A microcentrifuge at 24 ^oC. The supernatant was aspirated and the cell pellet counted with a Packard model 5650 gamma counter at 85% effeciency. Degradation of labelled ligand was measured by determination

of hormone precipatability with 5% trichloroacetic acid and comparison of ^{125}I-ligand with and without cells over a 2 hour time course. For determination of non-specific binding, a partially purified, insulin free preparation containing 8 ug/mg weight IGF-II, 50 ug/mg weight of insulin and 20ug/mg weight of IGF-I was employed.

In experiments involving receptor antibodies, membranes were first preincubated with various concentrations of antibody for 1 hour at room temperature prior to the addition of iodinated ligands. After the incubations, membranes were washed in 2 volumes of cold buffer and centrifuged. Radioactivity was determined as described above.

Affinity Cross Linking and SDS-PAGE

For each gel lane 50 ug of membrane protein was incubated with 10nM 125-I-IGF. After washing, the ligand was cross linked with 0.2mM disuccinimidyl suberate at 0^{o}C for 15 minutes. The reaction was quenched by the addition of 4 volumes of 50mM Tris-HCl, 1mM EDTA pH 7.4. The pellet was solubilized in 2% SDS, pH 7.0 with 1 % glycerol and 0.01% bromophenol blue, and electrophoresed on a 6.0% separating gel in the presence of 2M dithiothreitol or 5% v/v 2-mercaptoethanol as a reductant. Densitometric analyses of autoradiograms were performed on an Ultrascan XL laser densitometer (LKB Stockholm).

Vector for Genomic Library

The vector used for preparing the genomic library was Lambda FIX (Stratagene, La Jolla, CA). This vector is a lambda based vector similar to EMBL3, which accepts BamHI ligatable ends, and holds inserts with a size range of 10-22kb. It has T3 and T7 promoters on adjacent sides next to the cloning sites. Partially digested pieces of DNA (with MboI) were treated with Klenow to fill in two of the four bases, leaving a 2 base overhang compatible with the partially cut and Klenow treated insert. Calf alkaline phosphatase is no longer needed, and self ligation of the phage is blocked.

Microbial Cells Used

K802, a strain of E. coli, commonly used to propogate bacteriophage vectors and their recombinants, was used to infect the phage, amplify the genomic library, and screen the library. It is an su+ strain that is hsd R^-, hsd M^+, gal^- , met^- , sup E. . K802 was cultured
and grown on NZYM media and agar.

Probe Used to Screen Library

A 545 bp Eco R1- Bam H1 linker fragment from the rIGF-II cDNA clone 27 was used to make a probe for screening

the genomic library. In the cDNA clone 27 (see Fig 2), the Bam H1 site is located one nucleotide downstream from the ATG initiator, and an Eco R1 linker is ligated to its 3' end, 3 nucleotides downstream from the TGA terminator (Soares M.B.et al., 1983). To generate probes this had been subcloned into the Bam HI- Eco RI sites of the pGEM-1 vector polylinker. This vector was provided by Prof. Esfratiadis , Columbia University, New York . The fragment was labeled with ^{32}P using the random primed synthesis kit, as described below , as directed by the supplier (Boehringer Mannheim, Indianapolis, IN).

Preparation of Labelled Probes.

DNA was routinely labeled to specific activities of $0.5 \times 10^8 - 1 \times 10^9$ dpm/ug using (3000 Ci/mM, New England Nuclear, Boston, MA). DNA fragments for labelling were isolated by cutting the plasmid (in which the 545 bp rIGF-II cDNA fragment was subcloned) with Eco RI and Bam HI, and then separating the 545 bp piece on a 0.8% agarose gel. Tris acetate buffer (TAE: 40mM Tris Acetate, 2mM EDTA pH 8) was used. A slit was cut in the gel just ahead of the 545 bp fragment of interest and a DEAE membrane (Type NA 45 S&S) inserted. The gel was furthur electrophoresed for 30 minutes at twice the standard running voltage, and the DNA bound to the DEAE membrane was eluted and purified as outlined by the supplier.

Construction and Screening of The Genomic Library

The genomic library was made by Stratagene, San Diego, CA. The DNA for making the genomic library from the 18-54, SF cells was prepared by treating DNA with Proteinase K, dialysis and gentle phenol/chloroform extraction. The DNA was partially cut with Mbo1, and then run on a sucrose gradient (10-40%) to obtain approximate 20 kb fragments. It was then treated with Klenow to fill in 2 of the 4 bases, leaving a 2 base overhang to permit ligation with the vector, as mentioned above. The recombinant DNA so obtained was incorporated in the packaging extract Gigapak Gold (Stratagene, CA) to obtain recombinant phages. The library was then amplified to obtain a titer of 10^{10} pfu/ml.

To screen the library, overnight cultures of K802 E. coli cells were infected with the proper concentrations of phage, and plated on 150 mm NZYM agar plates such that about 20,000 discrete pfu were obtained after about 7 hours of growth. A total of 1×10^6 recombinant clones was examined for the presence of rat IGF-II specific sequences, using the 545 bp ^{32}P labelled rIGF-II cDNA probe mentioned above. The dishes were refrigerated for 2 hours to harden the top agar, and transferred for 5 minutes on to nitrocellulose. Duplicate transfers were made for 7 minutes. A sharp pin dipped in ink was used to prick holes through the paper and agar to mark the position

of the plaques. The DNA transferred onto the nitrocellulose membrane was denatured by soaking in 1.5 M NaCl + 0.5M NaOH for 2 minutes, neutralized in 1.5 M NaCl in 0.5 M Tris HCl pH 8 for 5 mins., and quickly rinsed in 0.2M Tris.Cl pH 7.5+ 2X SSC. The filters were then blot dried and baked at 68°C for 2.5 hours. Ten filters were stacked on top of each other and prehybridized in 20 ml of buffer (10X PIPES, 50% deionized formamide, 2% SDS, 0.1ug/ml denatured salmon sperm DNA), in 150mm dishes at 42°C for 2 hours. The buffer was decanted and 10 ml of fresh buffer was added, this time containing 10^6 cpm/ml buffer/filter. This was left overnight at 42 °C. The filters were washed under stringent conditions, with shaking, in 0.1 X SSC for 3 hours at 65°C, with three changes of washes. They were exposed overnight to XAR-5 33 X 40 cm film and developed. Several putative clones were obtained by such primary screening. The 'clones' were picked and rescreened (secondary screening) as above, except on 100 cm plates. After tertiary and quarternary screenings, a single recombinant phage containing 20 kb was isolated, and phage DNA was prepared.

Preparation of Phage DNA

To obtain small amounts of lambda phage DNA for mapping, a modified procedure (Grossberger, 1987) using ultracentrifugation of lysates was used. A positive plaque was picked up with a sterile 200 ul Eppendorf pipet and

transferred to a 14 ml glass tube containing 0.3 ml adsorption buffer (10 mM magnesium chloride and 10mM calcium chloride). To it was added 0.2 ml of an exponential culture of bacteria grown in L broth containing 0.4% maltose. The tubes were incubated for 10 minutes at 37°C, and 10 ml L Broth containing 10mM magnesium chloride and 0.1% glucose was added. The tube was shaken overnight at a 45 degree incline, with the cap in a half opened (loose) position. The tubes were centrifuged at 2000 rpm for 10 minutes to pellet the bacterial debris. The supernatant was centrifuged in a SW 41 rotor (Beckman) at 30,000 rpm for 30 minutes. The pellet was suspended in 200 ul of SM (10 mM Tris.Cl pH 8; 100 mM NaCl; 1mM EDTA pH 8; 0.2% $MgSO_4$), and transferred to a 1.5 ml microfuge tube. 200 ul of a freshly prepared solution of 1 mg/ml proteinase K in SM was added to each phage suspension, and the mixture was incubated for 2 hours at 37°C. The digest was extracted once with phenol and once with chloroform. The DNA was precipitated with 100 ul 7.5 M ammonium acetate and 1 ml 100% ethanol. The visible precipitate was then centrifuged immediately, washed with 100% ethanol, dried, and dissolved in 100 ul TE.

Restriction Analysis.

Restriction maps were determined by single and double digestions on the entire phage DNA. Locations of the restriction sites were determined by running restriction fragments on a 0.8% agarose gel, and blotting the bands

onto nitrocellulose, i.e Southern blots. Chromosomal DNA was also analysed using the method of Southern (1975).

Southern Analysis

About two microgram aliquots of restricted phage DNA, or 5 ug of total genomic DNA (restricted), were loaded per well on 0.8% agarose gels (Sigma), prepared with 1X Tris Borate EDTA buffer (TBE: 89mM Tris-HCl, 89mM Boric Acid, 1mM EDTA pH 8.0). Gels were electrophoresed overnight at 20 mA in 1X TBE buffer containg 0.5 ug/ml ethidium bromide. Lambda DNA restricted with Hind III was used in at least one lane as a molecular size standard. After electrophoreses, the gels were immersed in a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 2 hours, with gentle shaking. They were then transferred in neutralizing buffer (1.5 M NaCl, 1M Tris-HCl pH 7.5), and left there for 2 hours. DNA was transferred to nitrocellulose (type BA-85, 0.45um pore size, S&S) membrane by capillary action and then bound to the filter by baking in vacuo at 80 ^{o}C for 2 hours.

Filters were prehybridized at 42^{o}C for 2 hours inside heat sealable freezer bags. The rehybridization solution contained 2X PIPES buffer (Sigma), 50% deionized formamide, 0.2% SDS and 250 ug/ml sheared denatured Salmon sperm DNA. After removal of the prehybridization buffer,

filters were hybridized overnight at 42°C with hybridization buffer containing about 1-4 million cpm of 32-P labelled DNA probe. (The hybridization solution was identical to the pre hybridizing solution). Filters were washed at 68°C for 3 hrs with 0.1X SSC and 0.1% SDS , with changes in preheated solution every hour. These were then air dried and covered with plastic wrap, placed in film cassettes with Kodak XAR-5 film, and exposed at -70°C with DuPont Kronex Lightning Plus intensifying screens. Fragment sizes were determined from autoradiograms by measuring the migration distances.

The probe used for Southern analysis was the SalI-SalI fragment of 2.9 kb, as indicated in Figure 26.

DNA Sequencing.

DNA fragments from Sal I digests were cloned into bacteriophage M13mp18 and mp19 (Yanisch-Perron et al., 1985) in preparation for DNA sequencing. Double stranded RF phage DNA was prepared from 50 ml cultures of infected JM101 by using a scaled up version of the rapid isolation procedure described above. Pst I restricted phage and plasmid DNAs were joined with T4 ligase using a 3:1 target to vector (phage) ratio. After overnight incubation at 4°C, ligation reactions were mixed with competent E. coli JM101 and allowed to incubate at 0°C for 30 minutes. After 2 minutes at

42°C, 3 ml of molten top agar was added to each tube, followed by 10ul of 100mM isopropyl thio beta D-galactoside (IPTG, Sigma) and 50ul of a 2% solution in dimethylformamide of 5-bromo,4-chloro,3-indolyl, beta-D-galactopyranoside (X-gal, Boehringer). The mixture was poured into prewarmed, thick YT plates and after solidification, and incubated overnight at 37°C. White plaques from recombinant phage were stabbed with a sterile toothpick which was then placed in tubes with 5ml of 2X YT and 0.2 ml of log phase JM101 cells. The tubes were incubated at 37°C on a culture roller for 5-6 hours, and were used to isolate double and single stranded phage DNA.

Sequencing of specific fragments was done by the method of Sanger et al. (1977). Deoxy and dideoxy nucleotide triphosphates and M13 17-mer sequencing primers were obtained from Pharmacia (Piscataway, N.J.). Solid nucleotides were dissolved to give 10mM stock solutions as described by Maniatis et al., 1972, and stored at -20°C. Mixtures of stock solutions were made according to a Stratagene protocol for sequencing using ^{35}S dATP (400Ci/mmole NEN). The optimized nucleotide mixtures were: 25ul G + 25 ul 0.3 mM ddGTP; 25 ul A + 20 ul 0.1mM ddATP ; 25 ul T + 20ul 0.5mM ddTTP; 30 ul C + 20 ul 0,1mM ddCTP. Primer annealing was done in 1.5 ml tubes using 5ul ss DNA template, 1ul of primer, and 1.5 ul of 10X Klenow buffer (100 mM Tris pH 8.5, 100mM Mg Cl), in a total volume of 10 ul. This mix was incubated in a boiling water bath for 10 minutes and allowed to cool slowly

(20-30 minutes) to room temperature . 15 uCi of ^{35}S-dATP and 1-2 units of Klenow enzyme were added and the mix distributed into 4 aliquots of 2.5 ul each in 1.5 ml tubes. Reactions were initiated by placing 2ul of the appropriate N /ddNTP mix on the rim of each tube, and centrifuging in a microcentrifuge at 37^{o}C. Reactions were chased for 15 minutes with 2ul aliquots of 0.5 mM mixture containing all four dNTPs. Reactions were stopped by adding 4 ul of formamide dye mix (0.03% xylene cyanol, 0.03% bromophenol blue, 20mM EDTA in deionized formamide) and stored at -20^{o}C or loaded onto sequencing gels.

Polyacrylamide gels for DNA sequencing were composed of 6% acrylamide with 8M urea in 1X TBE Buffer. Gels were prepared by mixing 33.6g urea, 12 ml of 40% acrylamide (19:1 acrylamide: bis-acrylamide) stock, 16ml of 5X TBE buffer, and water to a final volume of 80 ml. Immediately prior to casting, 0.5ml of 10% freshly prepared ammonium persulfate and 45ul of tetramethylethylenediamine (TEMED, Kodak) were added to start the polymerization reaction. Each gel was cast between two glass plates (33 X 40 cm; one plate is 1.5cm shorter), separated by 0.4 mm plastic spacers (BRL, Bethesda , MD). Edges were sealed with plastic packing tapes, and plates were held together with binder clips. Before assembly, each plate was cleaned with deionized water, 95% ethanol, and acetone, in that order. The smaller plate was siliconized by wiping it with 2-3 ml of 2%

solution of dichlorodimethylsilane in chloroform, followed by evaporation in a fume hood for 10 minutes. After assembly of the plates and sealing, 60ml of acrylamide gel was drawn into a 60ml plastic syringe without a needle and squeezed slowly between the plates, which were tilted at a 30 degree angle. When the cavity was full, the flat side of a sharks-tooth comb (BRL, Bethesda MD) was inserted on the top of the gel to give an even surface upon polymerization. Gels typically were left horizontal for 1 hour for complete polymerization. The comb was then removed and the gel stored in an autoclave bag with moist paper towels at room temperature for up to 1 week.

Gels were set up by removing the bottom spacer and inserting the pointed side of the sharkstooth comb in the top of the gel. The comb was positioned so as not to puncture the top of the gel but to rest against it. The gel was clamped to a lucite gel stand and 1X TBE was added to the lower and upper reservoirs. After removal of any air bubbles at the bottom of the gel, a gel surface thermometer was attached, and the gel was prerun for 30-60 min. at 1200V, 50mA (EC high voltage power supply). When the gels reached running temperature (50-60^{o}C), sequencing samples were heated to 95^{o}C for 3 mins, and sample wells were rinsed out with 1X TBE from a squirt bottle to remove any urea that might diffuse from the gel. 2ul samples were loaded between the plastic teeth using a Hamilton microliter syringe with a plastic needle. All samples were loaded within 10 minutes.

Electrophoresis for 1.5 hours at 1200 volts was often followed by a second loading of the same samples to increase the resolution of large DNA fragments. All runs were stopped when the bromophenol blue dye reached the bottom of the gel. The gels were then disassembled and fixed in 10% acetic acid, 10% methanol for 20 minutes, followed by vacuum drying at 80^{o}C for 1.5-2 hours on a slab gel dryer . The dried gel was then filmed using XAR-5 33X 40 cm film in a large cassette for 1-2 days. Films were developed manually by immersion for 5 minutes in Kodak GBX developer, followed by 5 minutes in Kodak rapid fixer and hardener. The developed film was read on a light box to provide better clarity.

RESULTS AND DISCUSSION

18-54, SF is a well differentiated rat cell line of neuroendocrine origin (Wilson et al., 1987) that grows rapidly in monolayer culture in the absence of serum. Since it responds to several exogenous polypeptides (Wyche and Noteboom, 1979), it provides the opportunity to perform in vitro, studies which may be directly related to in vivo events.

The primary purpose of these studies was to determine what special properties allow this cell line to grow in the absence of any exogenously added growth factors.

Effect of Exogenously added Growth Factors.

Preliminary studies with many agents known to stimulate cell proliferation in other cell lines failed to identify a role for any specific factor (data not shown). Among the substances tested was the peptide hormone insulin (Fig. 3). Insulin has been shown to stimulate growth in fibroblasts, hepatocytes, hepatoma cells, mammary carcinoma cells, and F9 embryonal cells (Gospodarovicz et al., 1974; Osborne et al., 1978; Straus, 1971). But in 18-54, SF cells, insulin failed to stimulate growth. In fact, as shown in Fig 3, it decreased cell numbers. Insulin like growth

factor I (IGF-I), a related peptide, did not show any effect on the growth of 18-54, SF cells. IGF-II, however, when added in concentrations of 1 and 10 ng/ml, caused a significant increase in cell number.

Effects of Increasing Concentrations of Insulin and IGFs.

Figure 4 shows the effects of increasing concentrations of rIGF-II and IGF-I on replication of 18-54,SF cells. Neither IGF-I nor insulin, at concentrations as high as 100 ng/ml or 1.4 X 10^{-8} M, had any effect on the cell number. In fact, insulin significantly inhibited increases in cell number. On the other hand, significant stimulation by rIGF-II was observed at concentrations as low as 4-6 ng/ml (5.7-8.6 X 10^{-10} M). The maximal effect was achieved at a rIGF-II concentration of 10 ng/ml. During the course of nine experiments, stimulation of cell number by rIGF-II averaged 96.1+ 7.8% (mean + S.E.M.). This suggested that IGF-II is a growth stimulator to the 18-54, SF cells, whilst IGF-I and insulin, although known to stimulate other cell lines, have no effect on the proliferation of this cell line.

Probable cross reactivities of the receptors

There are suggestions about probable cross reactivities with receptors, and these findings raise questions

concerning which receptor type is utilized. The two IGFs have 62% identity in amino acid positions as well as significant homology with insulin (Rinderknecht and Humbel, 1978), and they display great similarities in their overall organization and primary structures. Tashjian Jr. et al.(1970) identified insulin receptors in the brain and demonstrated that insulin could promote cell proliferation. Pituitary cells also possess insulin receptors, but only high hormone concentrations can stimulate cell proliferation. (Goodyer et al., 1984; Rosenfeld et al., 1984). In human skin fibroblasts, insulin appears to exert its growth effects not by interaction to its own receptor, but via the IGF-I receptor. Therefore, it was decided to investigate whether the 18-54, SF cells possess receptors for insulin and IGF-I and/or IGF-II.

PRESENCE OF INSULIN RECEPTORS.

Time and Temperature Dependance

125-Iinsulin specific binding was assessed at 37°C, 24°C, and 4°C over a 6 hour time period (Fig. 5). Specific binding at 37°C peaked in 15 minutes, and rapidly decreased to near zero within 30-60 minutes. At 24°C, specific binding reached steady state levels at about 60 minutes and remained somewhat constant for about 3 hours. In contrast, specific binding at 4°C did not reach the peak

obtained at 24°C until at least 5 hours of incubation. The assay was linear for cell protein within the range of 0.1-0.8 mg of protein or cell numbers (3 X 10^5 to 3.5 X 10^6/ml), data not shown.

Binding Kinetics

Various concentrations of 125-I- insulin were incubated with 18-54, SF cell membranes preparations at 24°C for 90 minutes. Specific binding was determined with a set of tubes containing micromolar concentrations of porcine insulin incubated in parallel as the non-specific binding parameter. The insulin receptor displayed complicated binding kinetics, in that two binding plateaus were observed. Specific binding represented about 40%-50% of total binding. Half maximal saturation was achieved at low ^{125}I-insulin concentration (1.25 ng/ml or 0.2 nM) (Fig. 6 inset). As the ^{125}I-insulin concentrations was increased beyond 3 ng/ml, a second, higher capacity binding parameter was observed. This binding is of lower affinity, and it saturates half-maximally at about 1.33 nM. Specific binding was stable in fresh cell membrane preparations that had been frozen for up to 96 hours at 96-100% , and in preparations that had been frozen and thawed up to three times.

Displacement of ^{125}I-insulin.

The displacement of 125-I-insulin binding over a

broad range of unlabeled insulin concentrations indicates that increasing the concentration of competitor to 5000ng/ml displaces 50% of the ^{125}I-insulin bound to cell membranes (Fig 7). Also, human and porcine insulins are equipotent for displacing 125-I-insulin from membrane binding sites. IGF-I and IGF-II were also found to displace ^{125}I-insulin, but less effectively than unlabeled insulin (Fig 8). IGF-I displaced about the same amount of ^{125}I-insulin as one half the concentration of unlabeled insulin. IGF-II had a much lower (200 fold) affinity for high affinity ^{125}I-insulin binding sites, as compared to unlabeled insulin. Given the structural similarities between the IGF-I and insulin receptors, it seems reasonable for IGF-I to compete, albeit at a lower affinity, for the insulin receptor. However, in the experiments described, IGF-I did not significantly compete with ^{125}I-insulin. The reason for this is not known.

Degradation analysis of 125-I-insulin incubated with 18-54,SF cells for up to 2 hours indicated that up to 37% of the 125-I-insulin is in native form, whereas 94% is native in control dishes (no cells, data not shown).

Specificity of the Insulin receptor.

Neither pituitary fibroblast growth factor (FGF) nor human prolactin (hPRL) competed for ^{125}I-insulin binding. Similarly, none of the hypothalamic peptides, EGF or NGF competed for the ^{125}I-insulin binding sites (Table II).

Down regulation of the Insulin receptor.

To determine whether insulin pre-incubation with 18-54,SF cells affected ^{125}I-insulin specific binding, the time course of various insulin concentrations for receptor binding activity was studied. The effects of addition of 0.1-100 ug/ml insulin to cell cultures were assessed after 18 hour incubations. The cells were then thoroughly washed and the membranes prepared. At 50 ug/ml insulin, a maximal effect on insulin binding (50% inhibition) was achieved (Fig 9). 100 ug/ml of insulin was then added, and specific binding was assessed on cell membranes prepared at various times. By 3 hours a maximal effect (50% inhibition) was observed.

Presence of IGF-I receptors.

Though extensive binding studies and characterization of the IGF-I receptor were not performed, preliminary data, (Fig.15), and studies done in other laboratories on the 18-54, SF cells, indicate that there are specific IGF-I receptors on the cell line.

PRESENCE OF IGF-II RECEPTORS.

Specificity

Table III shows the total and specific bindings of ^{125}I-IGF-II in competition studies using several sources, as well as with insulin, IGF-I and of factors such as EGF. These data show that specific binding is obtained only with MSA (rIGF-I), IGF-II isolated from the 18-54, SF cells, and synthetic IGF-II. Insulin does not bind to the IGF-II receptor. Similarly EGF, insulin A chain and insulin B chain did not bind specifically to the IGF-II receptor, indicating that the binding site/ receptor for IGF-II is very specific.

Displacement of 125-I-IGF-II with unlabeled insulin and IGFs.

Specific binding of IGF-II to the 18-54, SF cell membrane averaged 20%/20 ug cell protein/ml, in contrast with insulin and IGF-I binding of 0.2% and 2%, respectively. Displacement studies for the IGF Type II receptor showed that ^{125}I-IGF-II was readily displaced by unlabelled IGF-II, but not by biosynthetic IGF-I or insulin, in concentrations as high as 1 ug/ml and 10ug/ml, respectively (Figure 10). Scatchard analysis revealed a Kd of 1nM and approximately 2.5 ug/ml receptor/mg membrane for the high affinity site.

Crosslinking IGF-II with its receptor.

The IGF-II receptor showed no change after reduction on multiple SDS-PAGE analyses (Fig 11). Multiple bands M_r corresponding to higher than 170 K, in addition to the major band, were observed. These bands were readily displaced by IGF-II, but not insulin, suggesting that they represent either partially proteolyzed or variant forms of the IGF-II receptor. Similar patterns have been reported for the rat IGF-II receptor, despite the presence of multiple protease inhibitors present during preparation (Mottola et al., 1984). This finding could also be related to different levels of receptor glycosylation and processing (MacDonald et al., 1985), since the preparation used is not limited to surface membrane receptors.

Role of Insulin and IGF receptors.

All of the above data support the conclusion that the 18-54, SF cell line has specific high affinity receptors not only for insulin , but also for IGF-I and IGF-II. Since it was also observed that insulin is not mitogenic for the 18-54, SF cells, the question of insulin acting as a growth factor in these cells by binding to either its own, or to the IGF receptors, is not pertinent. Likewise, IGF-I, though known to initiate growth in other cell lines, and in spite of the fact that it has specific receptors in these cells,

appears also not to act as a growth factor in this system. Its exact role, if any, is not known.

The 18-54, SF cell system appears to possess at least two kinds of insulin receptors (type I or high affinity, half-maximal saturation at 0.2nM ; and type II or low affinity, half maximal saturation at approximately 1.33 nM). A two receptor model for insulin action on embryonic heart cells, which are also sensitive to IGF-II, has been proposed (Wheeler et al., 1980). The receptor specificity suggests that only insulin or an insulin like growth factor (IGF-I or II) competes at this binding site. Recently, an atypical insulin receptor isolated from human placenta that binds both IGF-I and IGF-II with high affinity was described (Jonas et al., 1986). These findings also suggest the presence of a heterogenous population of insulin receptors. Since insulin does not bind to IGF-II receptor sites, insulin at low concentrations (nM) added to 18-54, SF cell membranes binds exclusively to insulin receptors. At high concentrations (1.75×10^{-8} M or higher) insulin may be binding to a heterogenous population of insulin receptors (high and low affinity), and possibly also to the IGF-I receptors. Again, the function of insulin binding to these receptors is unknown.

The binding of insulin to 18-54, SF cell membranes also appears to be influenced by the metabolic status of the

cell. When whole cells were incubated with ^{125}I-insulin with and without competitor, no specific binding could be detected during time courses of up to 2.5 hours at 24^{o}C (J. Wyche, unpublished data). Since IGF-II is secreted by these cells (Tanaka. H. et al., 1985), it could compete for ^{125}I-insulin specific binding sites on the 18-54,SF cells. Hence, in intact cells, IGF-II could occupy the insulin receptor at extracellular medium concentrations of 10-50 ng/ml (Lee et al., 1986).

Studies on IGF-II as an Autocrine Growth Factor.

Going back to the question of how growth in these cells is regulated in serum free media, the possibility that IGF-II, which is secreted by the 18-54, SF cells, may act as a growth promoting agent/ growth factor was explored. Figure 12 shows the effects of daily media changes. In this experiment, cells plated in serum free media without subsequent medium change grew faster than cells grown in serum-free media in which fresh medium was replaced on a daily basis. In general, cells grown under conditions of daily medium change lagged about 24 hours, relative to control cells. It appears, therefore that secreted IGF-II stimulated cell growth.

Since IGF-II has a growth promoting effect on the 18-54, SF cells, a radioreceptor assay with rat placental membrane was performed to determine the amount of IGF-II in

the conditioned medium. Table IV shows the concentrations of rat IGF-II found in media conditioned by 18-54,SF cells. After plating cells at relatively low density (2 X 10^3 cells in 3 ml medium in 28 cm plastic plates) under either serum free conditions or in 0.5% in fetal bovine serum, cell numbers showed little increase over the course of a 4 day incubation (Fig. 13). At that point the cells entered a phase of rapid proliferation, reaching a plateau by day 6. The rIGF-II concentration changes paralleled the time course. In the experiment summarized in Table IV, maximal rIGF-II concentration (75 ng/ml by rat placental membrane radioreceptor assay) was attained by day 9. In similar experiments, rIGF-II concentrations ranged from 60-100 ng/ml. IGF-I levels were undetectable.

It was thus shown that 18-54, SF secretes IGF-II and builds up concentrations as high as 100 ng/ml in six days in the medium. However, given the ability of the 18-54, SF cells to synthesize and secrete IGF-II, it is somewhat surprising to see these cells respond to low concentrations of exogenous IGF-II (4-10 ng/ml). One reason might be IGF-II competition for ^{125}I-Insulin binding on the 18-54, SF cell membranes. This would cause the endogenous IGF-II secreted by the cells to occupy insulin receptor sites of the intact cell at extracellular medium concentrations that range from 10-50 ng/ml. Such binding could explain the identification of insulin specific binding which was best done on isolated cell membranes rather than on intact cells.

It is conceivable, however, that addition of rIGF-II to freshly plated cells at a time when media concentrations of rIGF-II are still extremely low (see Fig. 13), may be of particular importance in generating an early mitogenic response. Thus, the findings that this cell line possesses high affinity specific receptors for IGF-II and attains levels of rIGF-II in conditioned media that corelate well with the K_D of the IGF-II receptor in these cells (10^{-9} M/L), plus observations that similar concentrations of IGF-II are capable of stimulating a variety of metabolic effects in other cell lines (Heaton et al., 1980; Janeczko et al., 1984, Verspohl et al., 1984), lead to the possibility that IGF-II acts as an autocrine growth factor in the 18-54, SF cells.

Thus, data indicate that this continuous cell line possesses specific IGF-II receptors, produces IGF-II, and is capable of proliferating in serum free conditions. Rat embryo fibroblasts (Adams S.O. et al., 1983), and fetal rat myoblasts (Hill D.J., 1985), in primary or early passage have been shown to possess IGF-II cell surface receptors, produce IGF-II, and show a growth response to purified IGF-II. Similar findings were reported for neural embryonal carcinoma cells; however these results were inconclusive due to the apparent low IGF-II production (Nagarajan et al., 1985). Nissley et al. in 1977 have argued against an autocrine role for IGF-II in BRL-3A cells. Their studies, howev-

er, involved a subline of rat liver cells, BRL-3A2 , which does not produce IGF-II and does not proliferate under serum-free conditions. The observations presented here are consistent with an autocrine function for IGF-II in the 18-54, SF cell line, and indicate that the cell line may provide a unique in-vitro system for further studies of the interrelationship between IGF-II and its receptor.

IGF-II Binding to the Type II Receptor

Still, other investigations have suggested that IGF-II actions are mediated through either insulin or type I receptors. Massague et al.(1982) have proposed that the IGF-II stimulation of proliferation in H-35 rat hepatoma cells is mediated via the insulin receptors. In the same cell line, Kret and coworkers in 1987 have reported that IGF-II stimulation of tyrosine aminotransferase, amino acid transport and glycogen synthetase activities are also mediated via the high affinity insulin receptors. Again, in 1987, Ewton et al. have similarly shown that the IGF-II receptors in L6 myoblasts do not mediate either IGF-I or IGF-II stimulation of amino acid uptake, cell proliferation and differentiation, or inhibition of protein degradation. Furthermore, investigations employing specific anti-receptor antibodies, have generally not supported the hypothesis that the IGF-II receptor is capable of mediating the metabolic and mitogenic actions of IGF-II. Several studies (Shimizu et al., 1986) demonstrated that inhibition of binding to the

IGF-I receptor in human fibroblasts by a specific monoclonal antibody inhibits both IGF-I and IGF-II stimulation of DNA synthesis and cell replication. Shimizu et al. also reported that stimulation of amino acid uptake in cultured myotubes by both IGF-I and IGF-II was inhibited by monoclonal antibodies against IGF-I receptors. Using a monoclonal anti body directed at the insulin and IGF-I receptor kinase, Morgan and Roth (1987) blocked the ability of insulin, IGF-I and IGF-II to stimulate glucose uptake in TA1 mouse adipocytes. The strongest evidence against the ability of the IGF-II receptor to mediate rIGF-II stimulated actions comes from the use of a polyclonal antibody against the IGF-II receptor in H-35 hepatoma cells. This antibody inhibited by 70-90% the specific binding of ^{125}I-IGF-II to H-35 cells (which possess no IGF-I receptor), although it failed to inhibit insulin binding (Massague et al., 1982). However, pretreatment of H-35 cells with antibody did not block IGF-II stimulation of DNA synthesis, thus strongly supporting the hypothesis that IGF-II action in these cells is mediated via the insulin receptor. Yet, the possibility that IGF-II could act through its own receptor to alter its responses to other factors, cannot be ruled out.

It therefore became important to determine whether IGF-II acted via its own specific receptor in the 18-54, SF cells, especially since these cells possess receptors for insulin and IGF-I, as shown above, IGF-II binds weakly to the insulin receptor. Studies of this cell line by other

workers (Rosenfeld et al 1986), had already indicated that the IGF-II binding to the IGF-I receptors is weak, showing that IGF-II may not act by binding to those receptors. Anti IGF-II receptor antibody r-II-PAB-I from the laboratory of Dr. Ron Rosenfeld at Stanford University was used to study the effect of blocking the IGF-II receptor on the growth of 18-54, SF cells.

The effect of antibody on the binding of 125-I-IGF-II.

The effect of the polyclonal antibody r-II-PAB1 on the binding of 125-I-IGF-II to 18-54,SF membranes is shown in Figure 14. Greater than 50% inhibition of binding was observed at antibody concentrations of 50 ug/ml (3.3×10^{-8} M), with 80% inhibition at antibody concentrations of 400 ug/ml. Specificity for the IGF-II receptor was demonstrated by affinity cross linking (Figure 15). 18-54,SF cells possess classical IGF-I receptors, and binding of ^{125}I-IGF-I is inhibited by both IGF-I and insulin, but not by r-II-PAB1 (lanes 1-5). On the reduced gel used, one can see the 130 kD subunit, as well as the 260 kD alpha-alpha dimer of the IGF-I receptor. Neither of these bands is inhibited by r-II- PAB1.

On the other hand, binding of ^{125}I-IGF-II to the IGF-II receptor (240 kD) is not blocked by insulin, but is significantly inhibited by r-II-PAB1 (lanes 6-10). Lane 9 shows significant inhibition of both the major (240 kD),

and the minor IGF-II receptor bands. Of note is the finding that the faint binding of the 125-I-IGF-II to the Type I receptor is not inhibited by r-II-PAB1.

Effect of anti-receptor antibody on cell growth.

The effect of r-II-PAB1 on the growth of the 18-54, SF was studied. Figure 16(a) shows the effect of a 5 day incubation with r-II-PAB1 on 18-54,SF cell number. As depicted in Figure 16(b), 10ng/ml of rIGF-II promoted a 90% increase in cell number by 5 days. However, a significant reduction in cell number was observed at r-II-PAB1 concentrations of 25 ug/ml. At 50 ug/ml, r-II-PAB1 invoked a 70% decrease in cell number. When 10 ng/ml rIGF-II and 35ug/ml r-II-PAB1 were simultaneously presented for 5 days, the inhibitory effects of anti-receptor antibody completely overcame the stimulatory effects of rIGF-II.

The effects of 5 day exposures to increasing concentrations of r-II-PAB-1 are shown in Figure 17. Cells receiving 12 ug/ml r-II-PAB1 were indistinguishable from those receiving control IgG, and they grew as near confluent monolayers. However, at r-II-PAB1 concentrations of 24ug/ml or more, progressive inhibition of 18-54,SF cell number was observed. At concentrations of 48-60 ug/ml, only sparse, isolated cells were found. Trypan blue staining indicated that 18-54,SF cells exposed to the highest concen-

tration of r-II-PAB1 were more than 85% viable. To more closely investigate whether anti-receptor antibody was indeed inhibiting cell replication (rather than increasing cell death), r-II-PAB1 was added to sparsely plated cells either at the time of cell seeding or after 24 hours (to permit cell attachment). After 5-7 days, cell colonies were stained with methylene blue, and the numbers of colonies and cells per colony counted (Figures 18 and 19). As shown, r-II-PAB1 caused a modest decline, typically to 50% of control values, and to 60-80% of values for cells exposed to preimmune IgG. A more striking effect, however, was seen in the number of cells per colony. Although the numbers of cells per colony in the wells exposed to preimmune IgG averaged 106-122% of control values, those in wells exposed to r-II-PAB1 displayed a 68-76% decrease. When the total numbers of cells per well were calculated (number of colonies X cells per colony), cells exposed to preimmune IgG showed no reduction (Figure 19). On the other hand, when r-II-PAB1 was added at time 0, a 91% decrease was observed. When r-II-PAB1 was added 24 hours after cell plating, there was 86% reduction.

Figure 20 depicts the morphology of colonies developed from sparsely plated 18-54, SF cells in the presence and absence of r-II-PAB1. Control cells plated in preimmune IgG grew as monolayers, as shown in Figure 17, whereas colonies grown in the presence of r-II-PAB1 were

significantly smaller and rounder (Fig. 21). Initially, discrete cells within the colony were difficult to identify, although colonies slowly increased in size. By days 4-5, discrete cells could be identified on the peripheries of the colonies, and by day 7 the entire colonies contained visually discrete cells (Fig. 21). Detachment of cells on days 5-7 by trypsin-EDTA resulted in the appearance of discrete cells, with more than 85% viability by trypan blue exclusion. Addition of fresh r-II-PAB1 on day 5 completely inhibited furthur growth of the colony, while addition of control IgG had no effect (data not shown). Similarly, when sparsely plated 18-54, SF cells were exposed to each of four monoclonal antibodies which immunoprecipitate the Type-II receptor, but do not inhibit binding of ^{125}I-IGF-II, no reduction in either colony number or cells per colony was observed.

Thus, under a variety of culture conditions, r-II-PAB-I significantly inhibited replication of 18-54, SF cells. In densely plated cells, 50 ug/ml antibody involved a 70% decrease in cell number by day 5. Furthermore, r-II-PAB1 was capable of blocking the mitogenic effect of exogenous IGF-II. Similarly, in sparsely plated cells, r-II-PAB1 greatly inhibited cell replication. Colony formation was variably inhibited (20-50%), but the major finding was a marked reduction in the numbers of cells per colony (typically to less than 20-30% of those in control wells). A similar effect was seen, regardless of whether r-II-PAB1 was added at the time of cell plating, or 24 hours later.

Thus, the major effect of antibody was on neither plating efficiency nor cell viability, but rather on cell proliferation.

The blocking of IGF-II action by antibodies specifically directed at the IGF-II receptor provides strong support for the hypothesis that this receptor may be capable of mediating specific IGF-II actions in 18-54, SF cells. Interestingly, two recent observations are consistent with this hypothesis. Kojima et al.(1987) found that IGF-II potently stimulated both calcium influx and thymidine incorporation in EGF-primed competent BALB/c 3T3 cells. r-II-PAB1, which both immunoprecipitates and blocks IGF-II receptor in this cell line, stimulated both calcium influx and DNA synthesis. Similarly,Hari et al.(1987) have recently reported that a polyclonal antibody directed against the human IGF-II receptor mimics the ability of IGF-II to stimulate glycogen synthesis in HepG2 cells.

Yet, the studies with the 18-54, SF cells reported here cannot be interpreted as providing definitive proof of the functional role(s) of the IGF-II receptor. First of all, polyclonal antibodies were used, and this raises the possibility that the observed effects are due to antibodies against other cellular antigens. Secondly, the 18-54, SF cells are unusual, and their responses may not be representative. However, recent immunohistochemical studies

employing antibodies against the IGF-II receptor such as R-II-PAB1, have indicated the ubiquitous presence of these receptors in a wide variety of tissues and cells. The IGF-II receptor has been localized primarily to the Golgi complex, and secondarily to the cell surface (Valentino et al., 1987).

Adams(1983) demonstrated that there were abundant IGF-II receptors in fetal tissue potentially capable of mediating both anabolic and mitogenic actions under both *in vitro*, and *in vivo* conditions. Since rIGF and its mRNA are also high in the rat fetuses, the peptide has been proposed to perform a major role in fetal growth.

The IGF-II receptor is not similar to other growth factor receptors

The IGF-II receptor displays several structural features which distinguish it from other growth factor receptors (Fig. 22). These include an unusual extracellular domain comprised almost exclusively of 15 cysteine based repeats which show no primary structural relationship to the cysteine-rich regions found in the receptors for insulin (Ullrich et al., 1985), IGF-I (Ullrich et al., 1986), LDL, or nerve growth factor receptors (Johnson et al., 1986). As mentioned earlier, the amino acid sequence of the mannose-6-phosphate receptor (which participates in the delivery of enzymes to the lysosome) (K. Von Figura and

Hasilik 1986), was found to be 99.4% identical with the sequence of the human IGF-II receptor (Oshima et al., 1988). This indicates that the two receptors are identical. It raises the question of whether one receptor can mediate physiological roles as dissimilar as the metabolic responses to IGF-II and the lysosomal targeting of proteins. It is possible that the IGF-II/ Man-6-P receptor mediates the cellular responses to IGF-II but does not participate in lysosomal delivery of proteins. Alternatively, IGF-II binding to the Man-6-P receptor may not have physiological significance. If so, there could be another IGF-II receptor present on cells whose presence would be masked by the rather large amounts of the Man-6-P receptor. This hypothetical alternative IGF-II receptor could be a member of the same family as the insulin and IGF-I receptors (i.e have an intrinsic tyrosine kinase activity) and be responsible for mediating the metabolic responses to IGF-II. Although it is unusual for one receptor to perform two such dissimilar functions, there is precedence for a single protein binding and responding to two distinct ligands. For example, the receptor for the neurotransmitter acetylcholine also binds thymopoietin (a hormone that regulates thymocyte differentiation) with high affinity, and thymopoietin affects neuromuscular transmission (Venkatasubramanium et al., 1986). Also, the bacterial protein 'tar' protein can mediate additive and independant responses to two distinct ligands (aspartate and maltose) (Mowbray S.L. et al., 1987). Thus,

the IGF-II/ Man-6-P receptor may participate in both processes. Indeed, it is possible that the presence of both the Man-6-P and IGF-II binding activities in the same protein allows for integration of two distinct signals. For example, the IGF-II/ Man-6-P receptor might respond to membrane or circulating proteins containing Man-6-P as well as circulating IGF-II. The ability of Man-6-P to increase the affinity of its receptor for IGF-II (Roth et al., 1987), would allow these two signals to act synergistically. Such a network of growth regulating receptors interacting with carbohydrates and growth factors has been suggested (Feizi et al., 1987).

Proposed mechanisms for IGF-II action.

The location of these distinct ligand binding activities within the extracellular domain of the receptor also presents an intriguing puzzle. The number of Man-6-P binding sites is difficult to evaluate because a single lysosomal enzyme (the presumed physiological ligand for the receptor) contains several phosphomannosyl residues. Consequently, the binding of lysosomal enzymes to the receptor occurs with a much higher affinity than the binding of single Man-6-P molecules (von Figura et al., 1986). In common with the 150 kD CDM6P (cation dependent mannose-6-phosphate) receptor, the multiple repeats of the IGF-II/Man-6-P receptor may present a series of binding sites which interact to give high affinity binding.

This focuses attention on the only other extracellular domain, the region that displays homology to fibronectin. The Type-II structures of fibronectin, factor XII, and protein CDC-109 probably provide the structural framework for binding sites whose specificities are defined by unique residues within or adjacent to the Type-II region. For example, a unique 13-residue segment adjacent to the Type-II structure of fibronectin is crucial for its collagen binding activity. Thus, the IGF-II receptor combined with certain adjacant residues, could contribute to an IGF-II binding site. The ligand binding specificity and the antigenic structure of the IGF-II binding protein are similar to those of the IGF-II receptor, and its size is slightly less than that of the membrane associated receptor (Kiess et al., 1987). Thus, proteolytic cleavage could release a truncated receptor which might then act as a soluble IGF-II binding protein. Although a single mRNA was detected by Northern analysis (Morgan et al., 1988), it is also possible that alternative splicing of the receptor mRNA yields a truncated receptor. The serum transport protein and transmembrane receptor molecule may originate from the same gene.

Given its unusual structure and small intracellular domain, the IGF-II receptor could act via the following mechanisms. It could promote elevation of calcium levels which would transmit signals across the plasma membrane to

the cell's interior by acting as a gated channel that opens transiently in response to IGF-II or Man-6-P. The ligand could then travel to the lysosome, undergo cleavage and yeild products that could activate the cell to respond.

Although no attempt was made in the current study to correlate the two receptors (IGF-II and the Man-6-P), or to study the mechanisms of action, the findings seem to indicate that at least in 18-54, SF cells, the IGF-II/Man-6-P receptor does play a role in IGF-II mediated autocrine stimulation of growth .

Still, the finding that the IGF-II and Mannose 6-P receptors are related (if not identical) proteins, suggests new avenues of investigation. It also further compounds the puzzle of the physiological role of IGF-II and its receptor. Recombinant DNA has recently been produced, and DNA clones for IGF-II receptor are available (Morgan 1987; Furman 1987). These studies should facilitate the understanding of IGF-II gene regulation at the molecular level.

Genetic Studies of the rat IGF-II gene.

From studies in the rat liver , the rat IGF-II gene, like the human counterpart, has been shown to contain three coding exons and three promoters (Fig 23 and 24) (Frunzio et al., 1986). The structures and sequences of two of the promoters and their corresponding noncoding exons are

conserved between rodents and humans (Ueno et al., 1988). The most 5' rat IGF-II promotor differs from its human counterpart in relative location and nucleotide sequence, and additionally in its association with a single untranslated exon. Like the human gene, rat IGF-II resides immediately 3' to the insulin 2 gene, and it has the same transcriptional orientation. The relative location of the tyrosine hydroxylase has not been mapped in rodents, but in humans it lies next to the insulin gene (Dull et al., 1984). Like IGF-I, the IGF-II gene is transcribed and processed into several mRNA species.

The steps involved in generation of IGF-II mRNAs appear to be more complicated in the rat than in the human. Multiple RNA species ranging in size from 6.0 to 1.2 kb have been detected on Northern blots from rat tissues (Soares et al., 1986). Several of these species can be accounted for on the basis of alternative promotors (the 5.0 kb mRNA derives from the middle promotor, and the 4.0 kb species derive from the 3' promotor (Fig. 18). The others may be formed by variable RNA processing or polyadynelation at the 3' end of the gene, although the exact mechanism involved in the synthesis of these RNA species has not been determined. No rIGF-II peptides derived from differential processing have been described, and it appears that differential RNA splicing affecting the B domain coding region, specifically for serine 29 (Zumstein et al., 1985), may be an event limited

to the human gene. The marked nucleotide sequence differences between the human and rat genes near the putative variant splice acceptor sites probably account for the unique human pattern of RNA processing.

Tissue specific factors also play roles in IGF-II gene expression. Human and rat fetal liver, skeletal muscle and skin contain high levels of IGF-II mRNA, whereas hypothalamus cerebral cortex, brain stem and thymus contain relatively low levels (Han et al., 1988; Brown et al., 1986). As noted, in adult rat brain IGF-II gene expression is maintained, and the mRNA content far exceeds that of other tissues (Gray et al., 1987, Tricoli et al., 1986). A number of tumors appear to synthesize IGF-II mRNA constitutively. A partial list includes several embryonic tumors such as Wilms', neuroblastoma, hepatosarcoma, rhabdomyosarcoma, pheochromocytoma, leiomyosarcoma, colon carcinoma, and liposarcoma (Hoppener et al., 1988) .Several of these tumors synthesize IGF-II and IGF-I mRNA, as does rat medullary carcinoma. The relationship of IGF mRNA expression to IGF peptide synthesis, and the roles of IGFs in tumor growth or progression are not clear from these data, and there appears to be no specific tumor cell or tissue type in which either growth factor protein or mRNA is exclusively produced.

In any event, the exact mechanism of gene regulation is not known. Analyzing the IGF-II gene, and studying its sequence might provide some answers. Since

IGF-II in the 18-54, SF cells was shown to play a unique role, it would be interesting to study the makeup of the IGF-II gene in these cells.

Since other investigations have described the isolation mapping and sequencing of the rIGF-II gene (Frunzio et al., 1986; Soares et al 1986), it seemed appropriate to perform a similar study with the IGF-II gene in the 18-54, SF cells to see if it is regulated or made up in ways other than those found in other rat cell lines. It was decided to make a genomic library from the 18-54,SF cells DNA, which would enable isolation and study of the IGF-II gene in these cells. A piece of the cDNA of the rIGF-II gene was used as a probe (see Materials and Methods), to screen the genomic library. By plaque hybridization and on screening 10^6 clones, it was possible to isolate one clone (clone 22).

Restriction mapping of the IGF-II gene.

Restriction mapping was done on the recombinant clone 22, to see whether the genetic makeup of IGF-II from the 18-54, SF cells was similar to the gene described for other rat cells (Frunzio et al., 1987). The strategy was to use the restriction enzymes employed by others, and to determine whether the same length fragments are generated on Southern blots and identified with probes from the

coding and non coding regions, as described in Materials and Methods. Fig. 25 is an illustration of one such gel. As can be seen in Fig. 25, Eco R1 (which does not have a restriction site in the gene), generates a full length clone of 20 kb. Also, as expected, Xho 1 gives two fragments of 4.5kb and 3.5 kb, whilst Hind III gives the 9.0 kb band found in other studies. Pvu II generates 2.0 and 2.5 kb fragments. Although Figure 25 is just one example of the numerous gels obtained, it shows that the IGF-II gene in the 18-54, SF cells does not differ from that of other rat cell lines. Fig. 26(b) shows a detailed restriction map which is identical to those for IGF-II genes from other rat cell lines (Fig. 26 (a)). However, clone 22 was interesting because it contained an upstream 5' region that has not previously been isolated and hence studied. It also shows that clone 22 is complete, and that it contains all of the first, second and third promoter regions.

Figure 27 depicts the 5' sequence analysis from the M13 cloned SalI-SalI fragment. As can be seen from the sequence, there is an XbaI restriction site 309 bp from the start of the fragment. By restriction mapping, the XbaI site was 343 bp long, as indicated in Figure 27. This confirmed the accuracy of the clone. Also, since this gene has three different promoters, the 5' upstream region may provide a useful tool for studying regulation or regulatory signals.

Proposed mechanisms of IGF-II gene regulation.

Figure 28 shows the Southern blot of the total genomic DNA from 18-54, SF cells, adult rat liver cells and BRL-3A cells respectively, digested with Bam HI, Bgl II, and other restriction enzymes, as indicated. As the data show, for all the restriction enzymes used, except for Xho I and Sac II, the banding pattern for the 3 cell types is the same. This may be important because Xho I does not cut methylated DNA. In the blot, the band in adult rat liver cut with Xho I is absent. It may be that the gene is turned off in adult rat liver, but not in 18-54, SF and BRL-3A cells. Similarly, Sac II, an isoschizomer of Sst II, used to differentiate between methylated and unmethylated sequences, shows a band in adult rat liver, but not in 18-54, SF or BRL-3A cells, again indicating that the adult rat liver IGF-II gene may be turned off because of possible methylation. Thus, prelimnary findings suggest that methylation could be one method by which the gene is regulated. Very little is known so far about IGF-II gene regulation. Isolation of the upstream region and promoter region of the gene can facilitate studies of IGF-II gene regulation and expression.

In conclusion, it was shown in the above study that IGF-II plays a role as an autocrine growth factor to the 18-54, SF cells. Studying the IGF-II gene and isolating the

upstream region can facilitate studies of regulation and expression of this gene.

SUMMARY

The 18-54, SF is a rat cell line which grows under serum-free conditions, and secretes rat IGF-II into conditioned media.

(i) Media concentrations of rIGF-II as high as 75 ng/ml are achieved by confluent cells, and depletion by daily changes of conditioned media leads to growth attenuation.

(ii) Exogenous rIGF-II increases cell numbers wheras IGF-I and insulin have no effect.

(iii) This cell line also possess classical IGF-II receptors, along with receptors for IGF-I and insulin. The IGF-II receptors have now been shown to be identical to Man-6-P receptors.

(iv) Exposure of the 18-54, SF cells to an antibody against the IGF-II receptor decreases cell numbers, primarily via inhibition of clonal proliferation. The findings are consistent with an autocrine role for rIGF-II which is mediated through its own receptor.

(v) By screening the genomic library with a rat cDNA clone, the IGF-II gene from the cells was obtained along with the 5' upstream region that has not been studied so far. It was demonstrated that the gene does not differ from the rIGF-II

FIGURES

Figure 1.

Sequence homologies between human insulin , IGF-I and IGF-II.

Data represent the absolute number of amino acids common to the peptides listed, as well as the overall percent sequence identity.

(Taken from Rinderknecht and Humbel (1978)).

Fig 1 SEQUENCE HOMOLOGIES BETWEEN INSULIN AND IGFs

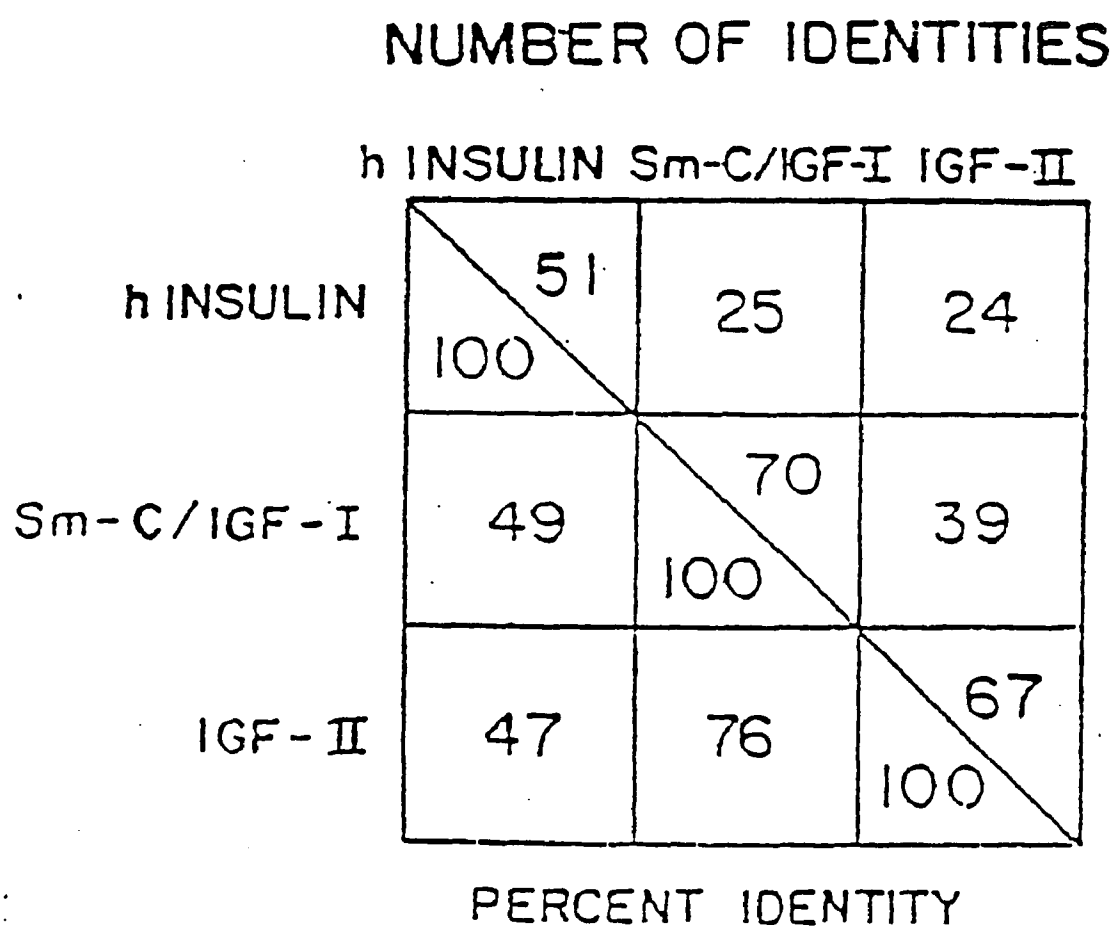


Figure 2

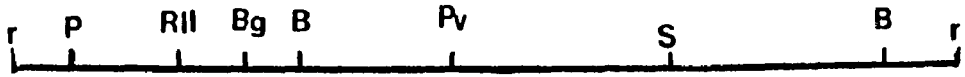
Probe Used to Screen Genomic Library and for Southern Analysis for Restriction Mapping.

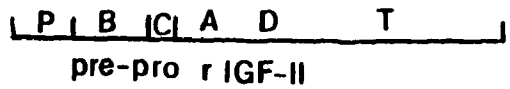
A. Restriction map of the pre-pro-rIGF-II cDNA (P=Pst, B=Bam, Pv=PvuII, RII= Eco RII and S=SacI). The region encoding the rIGF-II precursor is indicated in the second line. (P = preregion, domains B, C, A, and D of rIGF-II, and T = trailer polypeptide).

B. The 545 base pair cDNA probe used for restriction mapping and screening of the genomic library is shown.

Taken from Soares et al., 1985

A





B

Fig 2

Figure 3

The effect of insulin, and IGFs on 18-54, SF cell growth.

Cells (5 x 10^4 per 60mm X15mm dish), were incubated in serum free medium without and with insulin (1ug/ml), IGF-I (1 and 10ng/ml), IGF-II (1 and 10 ng/ml) for 5 days at 37°C. Cells were then trypsinized and counted. The bars represent the means of triplicate determinations from a single experiment _+ S.E.M (limits). The experiment was repeated three times with similar results.

Fig 3 EFFECT OF INSULIN AND IGFs ON CELL NUMBERS

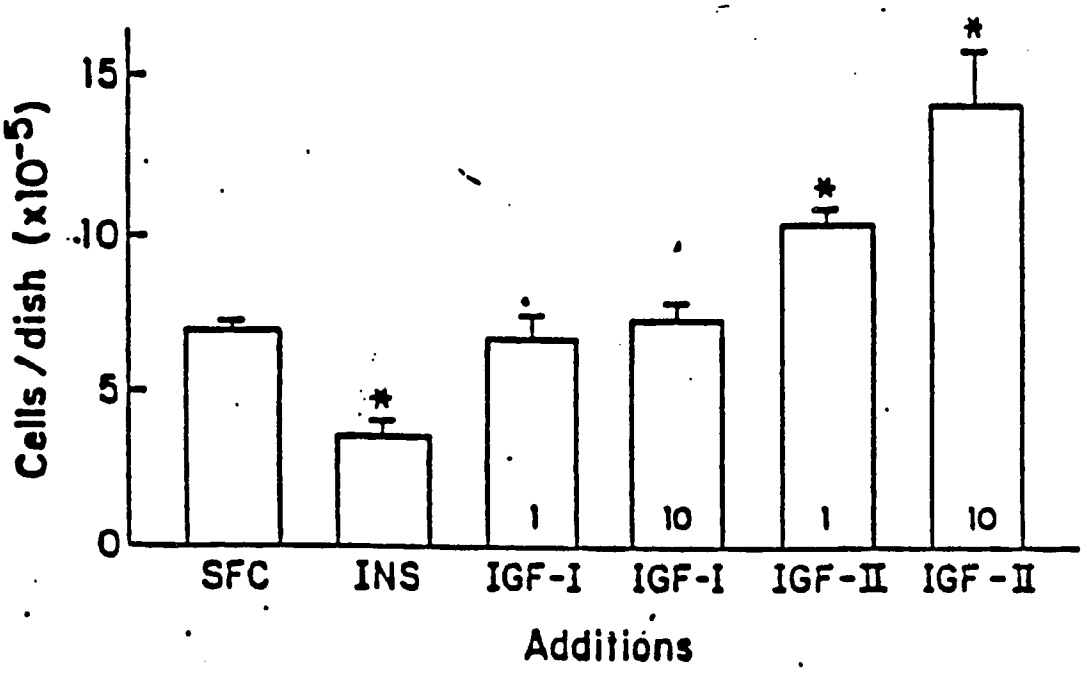


Figure 4

IGF-II Stimulation of Cell Replication.

18-54,SF cells (4.5×10^4) cells per 60mm x 10mm dishes) were plated in 5ml of serum-free medium. Sets of dishes run in triplicate were supplemented with either PBS or indicated concentrations of IGF-I or rIGF-II. Cell cultures were incubated ays, and then trypsinized and counted. Each point represents the mean $\pm$ S.D.

Fig4IGF STIMULATION OF CELL REPLICATION

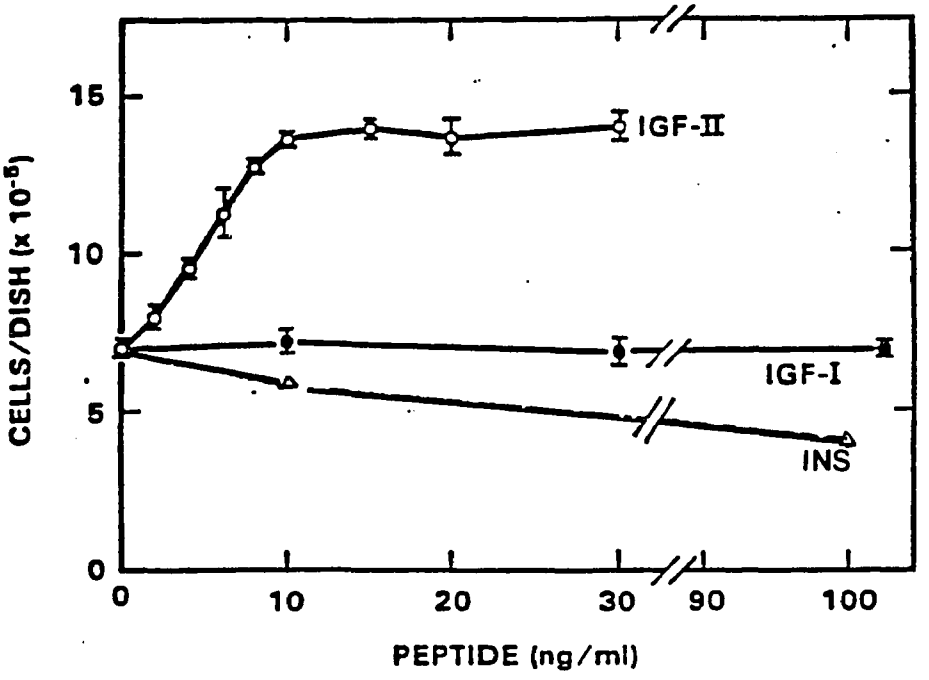


Figure 5

Time and Temperature Relationship of 125-I-insulin Binding to 18-54,SF cell membranes.

To membrane equivalents of 2 x 10^6 cells were added 0.8 ng/ml 125-I-insulin (in triplicate). To a parellel set of tubes was added 2ug/ml porcine insulin (non-specific binding). Tubes were incubated at 37°C (●), 24°C (O), and 4°C (▲). At various time points, tubes from both sets were removed and specific bindings determined.

Fig 5 Time and Temperature Relationship of ^{125}I-Insulin Binding

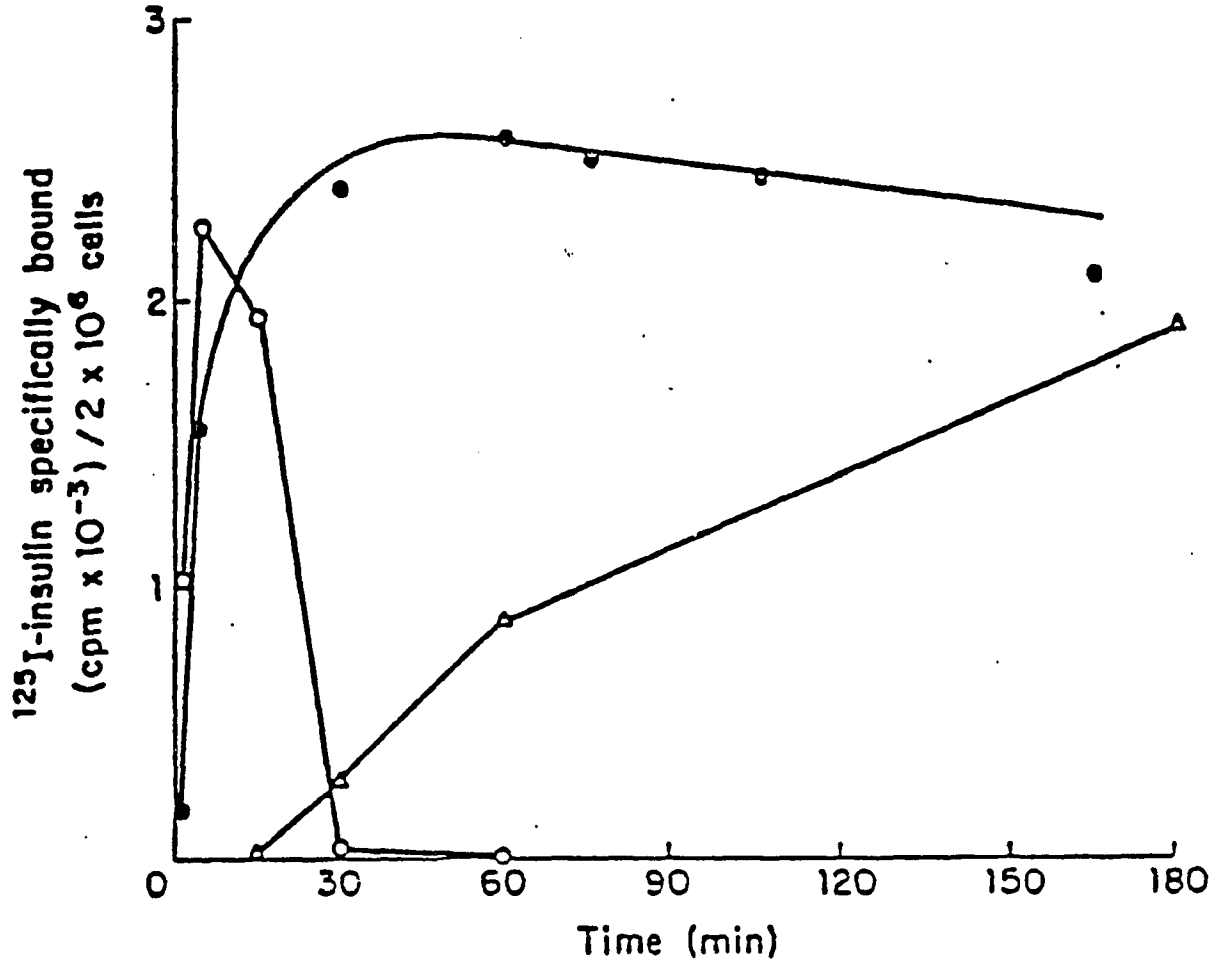


Figure 6

Binding of 125-I-insulin to 18-54,SF cell membranes.

Binding was measured in membrane preparations (100ug protein per assay tube) with and without a 100 fold molar excess of porcine insulin. The values reported are specific binding (approximately 35% of the total binding) at low concentrations (inset) and over a broader concentration range.

Fig 6 ^{125}I—Insulin Binding

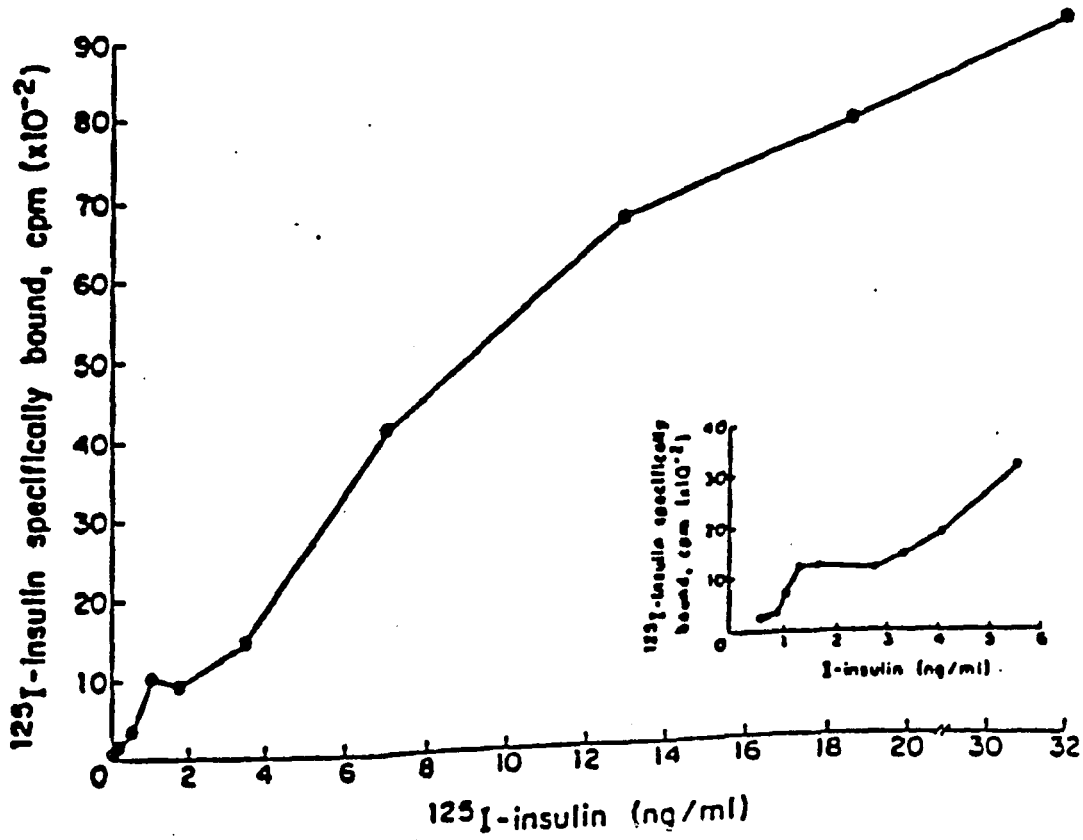


Figure 7

Inhibition of ^{125}I-Insulin Binding to 18-54, SF cell membranes by Insulin From Different Species.

1.5 ng/ml 125-I-insulin was mixed with 100ug cell membrane. To it was added 0-5000 ng/ml unlabeled human (O) or porcine (●)insulin.

Fig 7

DISPLACEMENT OF 125-I-INSULIN BINDING BY INCREASING CONCENTRATIONS OF INSULIN

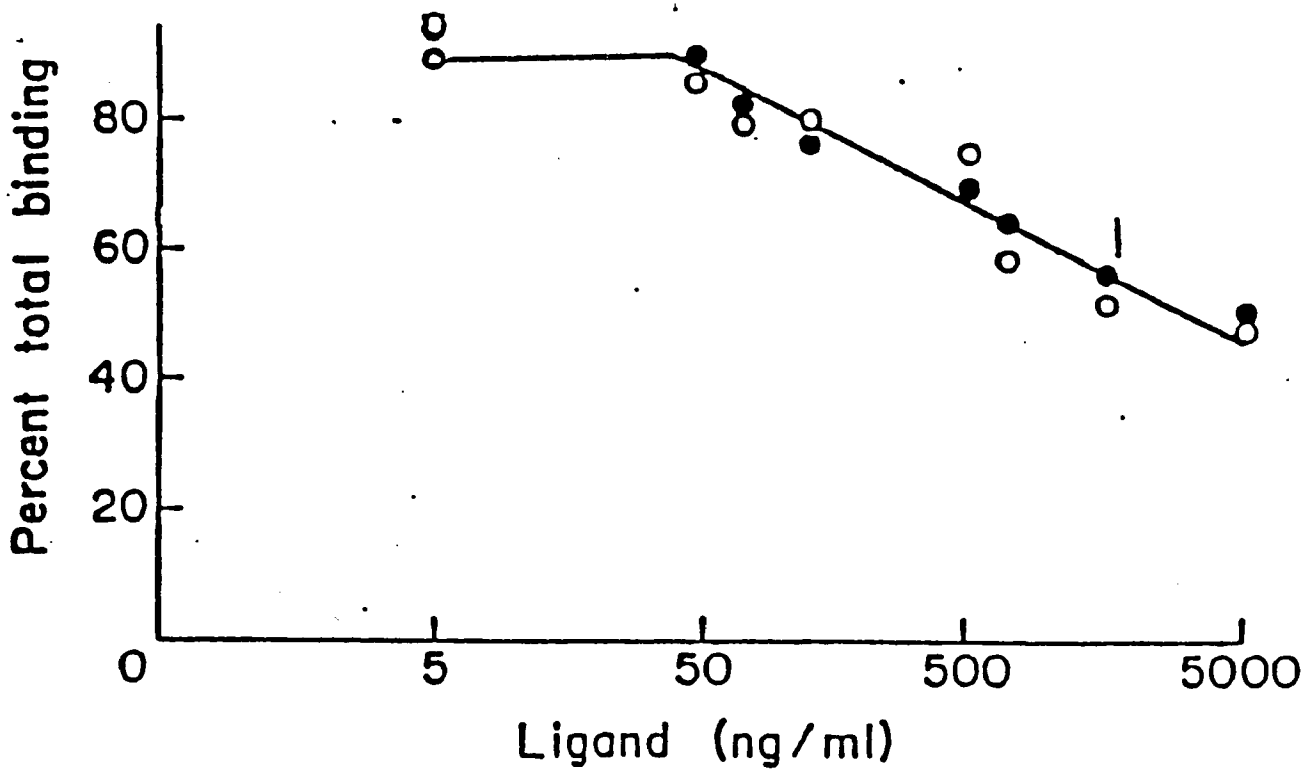


Figure 8

Inhibition of 125-I-Insulin Binding to 18-54, SF cell membranes by Insulin-like Growth Factors.

1.5 ng/ml 125-I-insulin was added to 100 ug cell membranes (total binding at 100%), plus 0-1000 ng/ml of unlabeled porcine insulin (●), IGF-I (Δ) or IGF-II (O).

Fig 8

DISPLACEMENT OF 125-I-INSULIN BINDING BY INCREASING CONCENTRATIONS IF-INSULIN AND IGFs

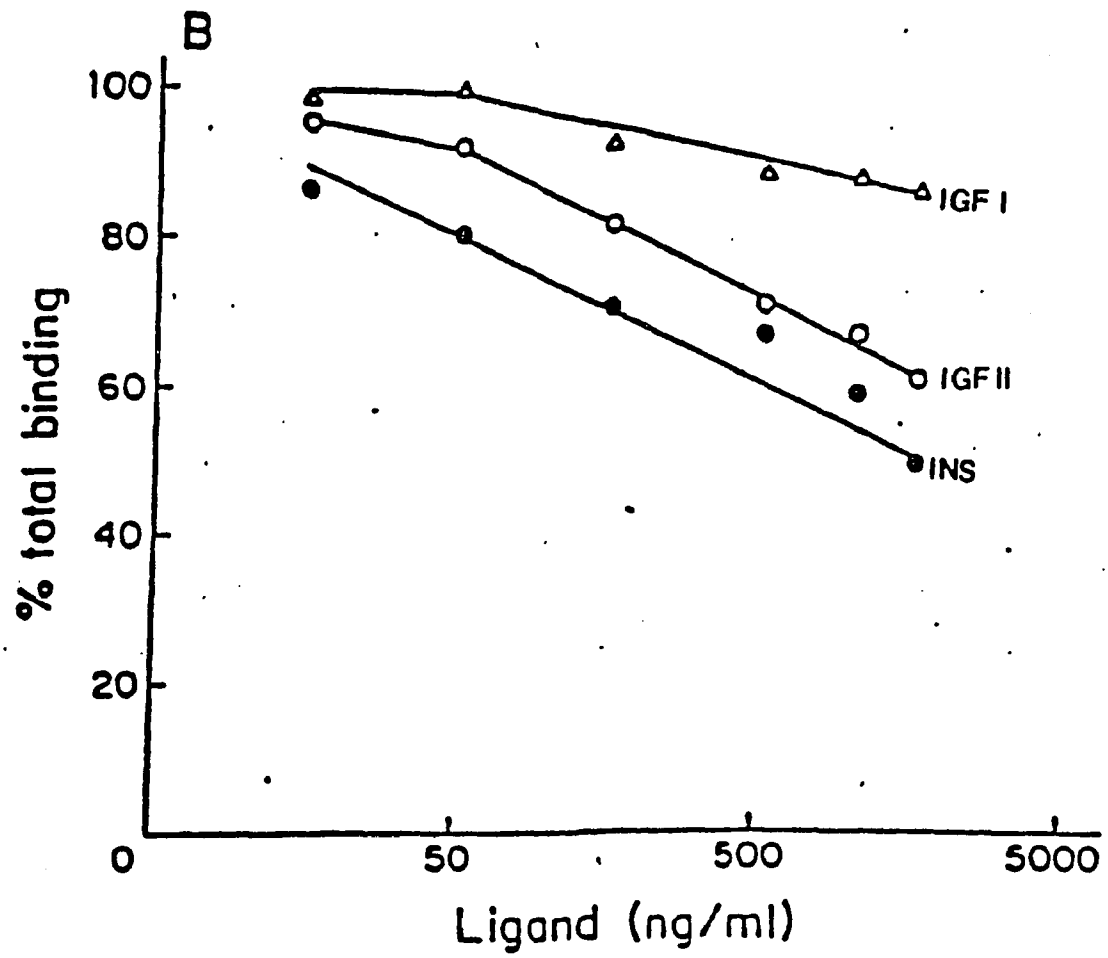


Figure 9

Insulin down-regulation of 18-54, SF cell receptor as a function of insulin concentration and time of incubation.

A. Cells (3×10^6) were incubated with the indicated concentrations of porcine insulin at 37°C for 18 hours. Cell membranes were prepared and 125-I-insulin specific binding determined.

B. 3×10^6 cells were incubated with 100 ug/ml of insulin for various times, samples prepared and specific binding determined. Each point represents the mean (S.E.M.)of six determinations of two different experiments.

Fig 9 DOWN-REGULATION OF INSULIN RECEPTOR

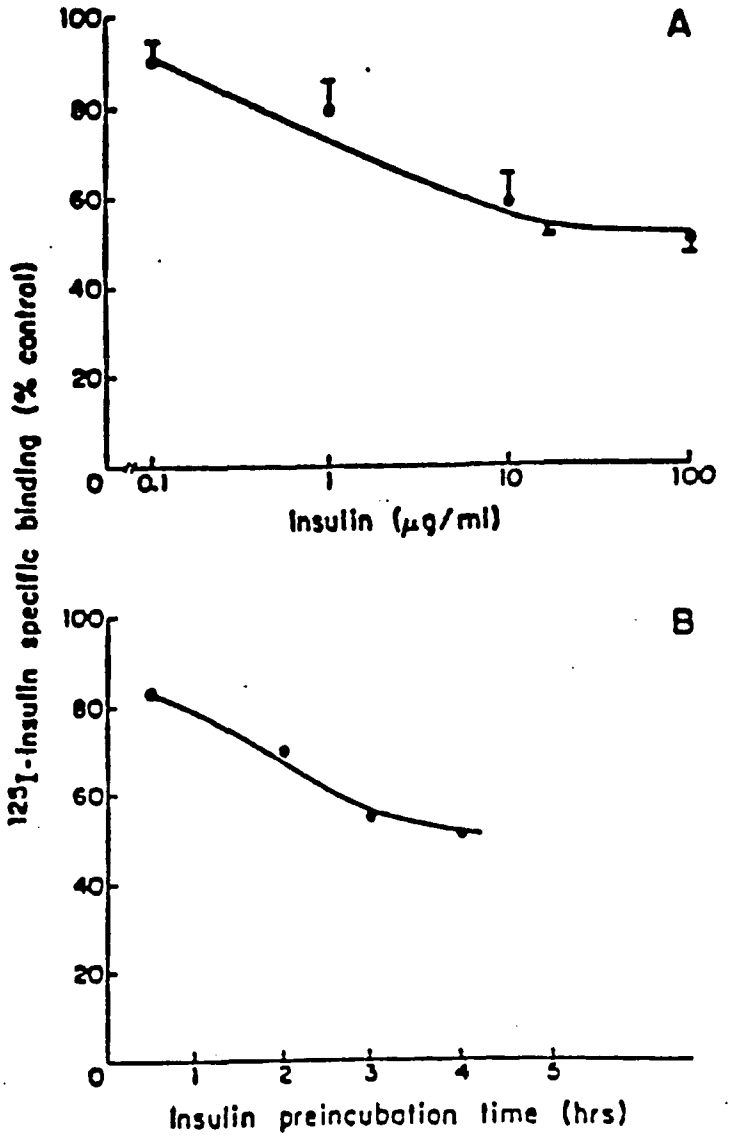


Figure 10

Displacement of 125-I-IGF-II binding by increasing concentrations of unlabeled IGF-II (●), IGF-I (O), or insulin (Δ) in 18-54, SF cells.

Fig 10
DISPLACEMENT OF 125-I-IGF-II BINDING BY INCREASING CONCENTRATIONS OF IGF-II

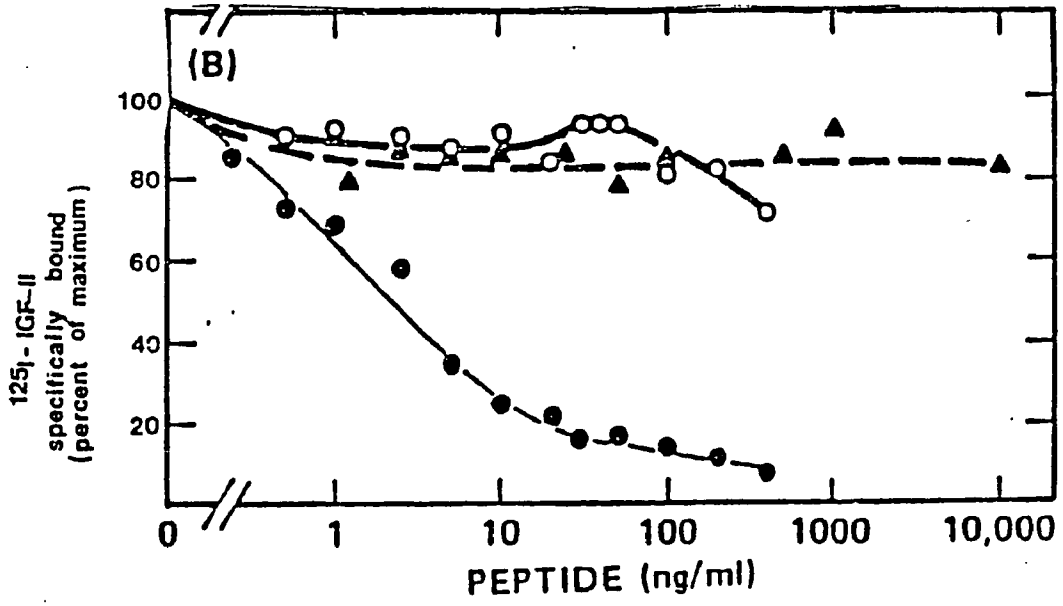


Figure 11

SDS-PAGE of the cross-linked IGF-II receptors on 18-54, SF cells.

Autoradiogram of 18-54,SF membranes (200ug) were cross-linked to 125-I-IGF-II. 6% separating gels were run in SDS and cross-linking was performed without unlabeled peptide (a), or in the presence of excess unlabeled IGF-II (b) or insulin (c). A unreduced; B reduced.

Fig 11 IGF-II BINDING TO 18, 54-SF CELLS

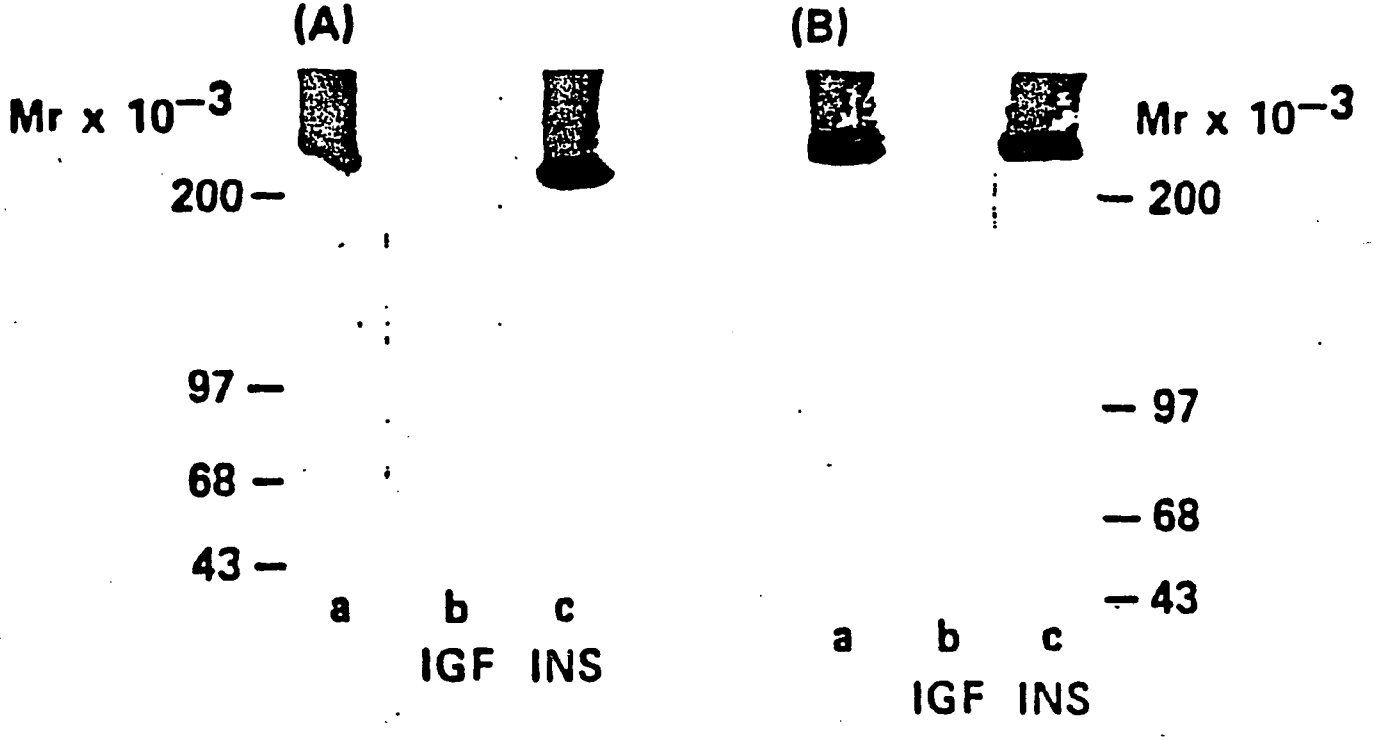


Figure 12

Effect of daily media change on cell number.

18-54, SF cells (5 x 10^4 cells per 60mm x 10mm dish) were plated in 5 ml serum free medium on Day 0. One set of dishes received no media change (O). The second set of dishes recieved daily media changes (O). Cells were trypsinized and counted with a cell counter. Each point represents the mean cell number from three dishes which did not vary by more than 10% of each mean value.

Fig12
EFFECT OF DAILY MEDIA CHANGE ON CELL NUMBERS

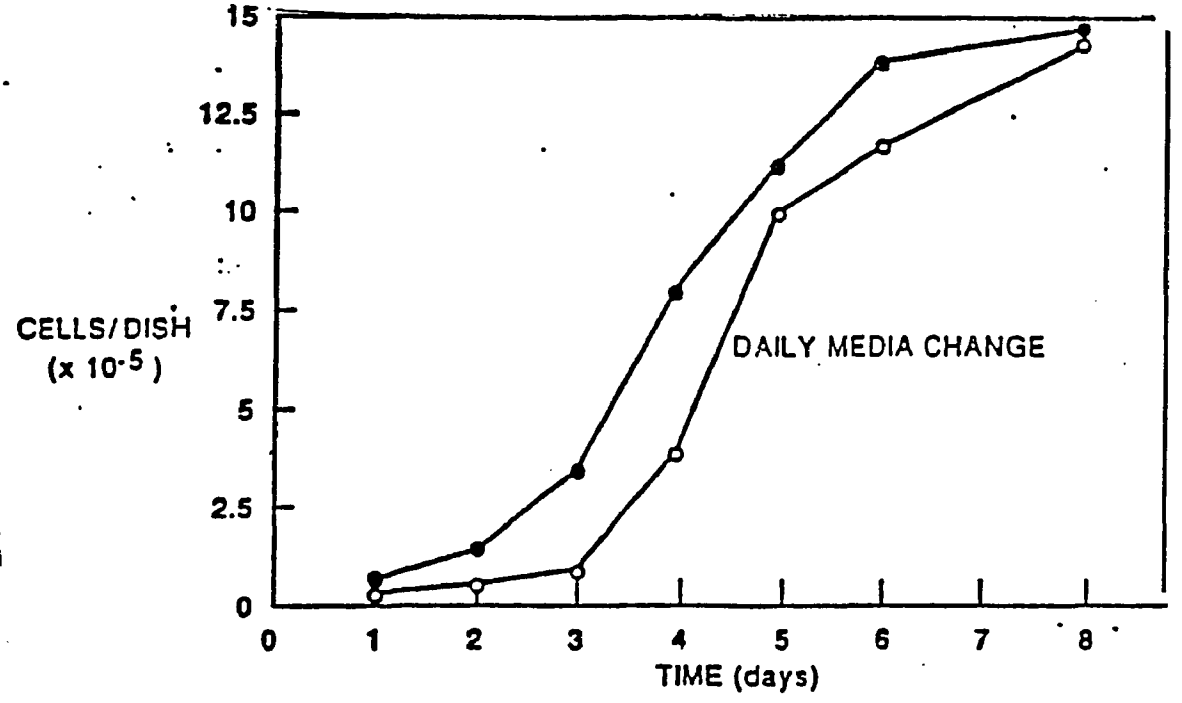


Figure 13

rIGF-II Concentrations in Conditioned Media

18-54, SF cells were plated at low density (2 x 10^3 cells in 3 ml medium in 28 cm plastic plates). The media conditioned by the 18-54, SF cells were subjected to radio-receptor assay to determine IGF-II concentrations, and to determine DNA by a flourometric method.

Fig13 18-54, SF: r IGF II PRODUCTION

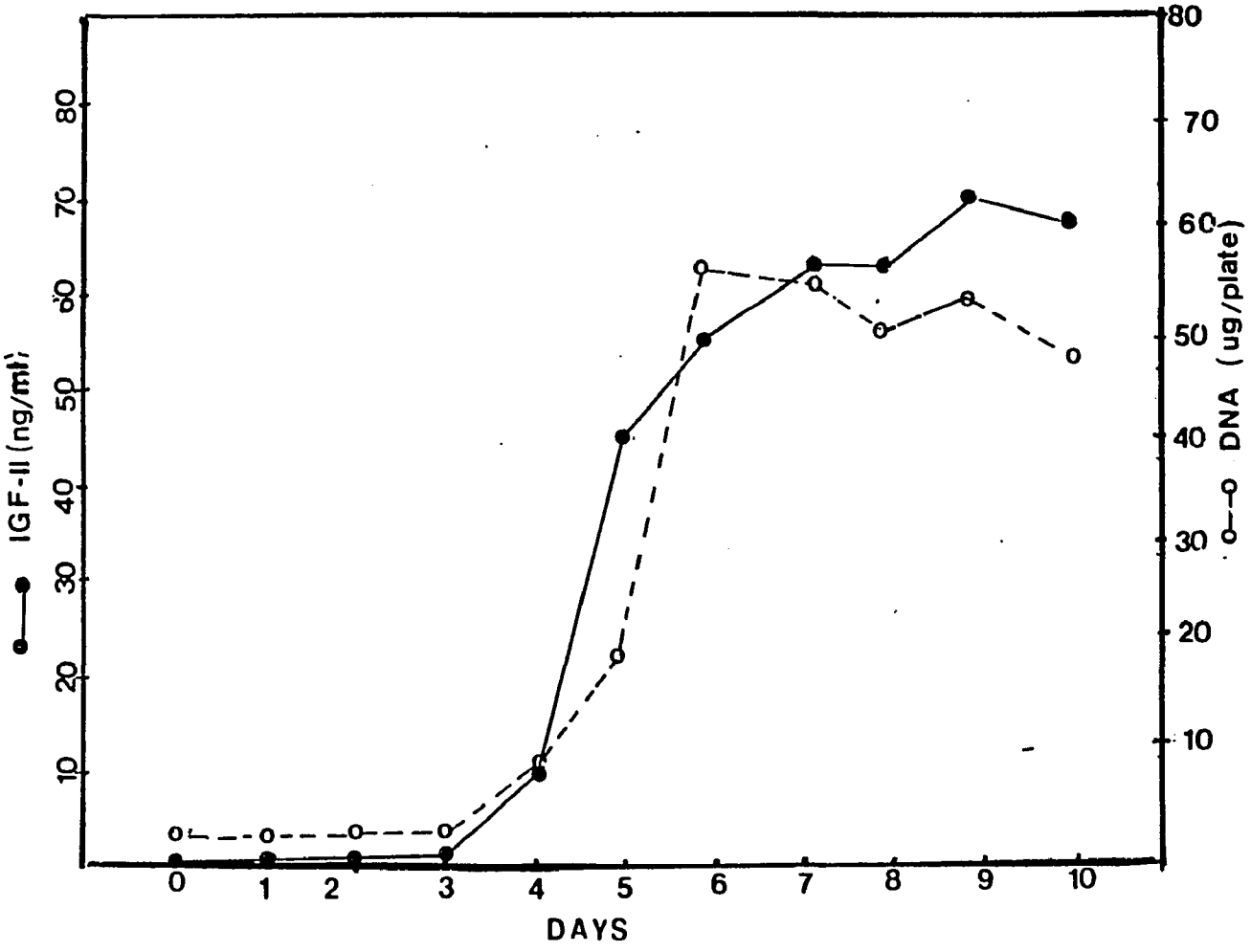


Figure 14

Effects of R-II-PAB1 on 125-I-IGF-II Binding to 18-54, SF cells .

Membrane preparations from 18-54, SF cells (100 ug protein) were suspended in 50mM Tris buffer, 0.5% BSA, pH 7.4, and preincubated with indicated concentrations of R-II-PAB1 or control IgG for 1 hour at room temperature. At the end of the pre-incubation period, 125-I-IGF-II was added, and membranes incubated for an additional 2 hours at room temperature. Subsequently membranes were washed in two volumes of cold buffer, centrifuged, and assayed for radioactivity . Non-specific binding was determined in the presence of excess unlabeled peptide, and subtracted from total binding to calculate specific binding. Points represent the mean+ S.D. of triplicate determinations.

Fig 14

EFFECT OF R-II-PAB1 ON 125-I-IGF-II BINDING

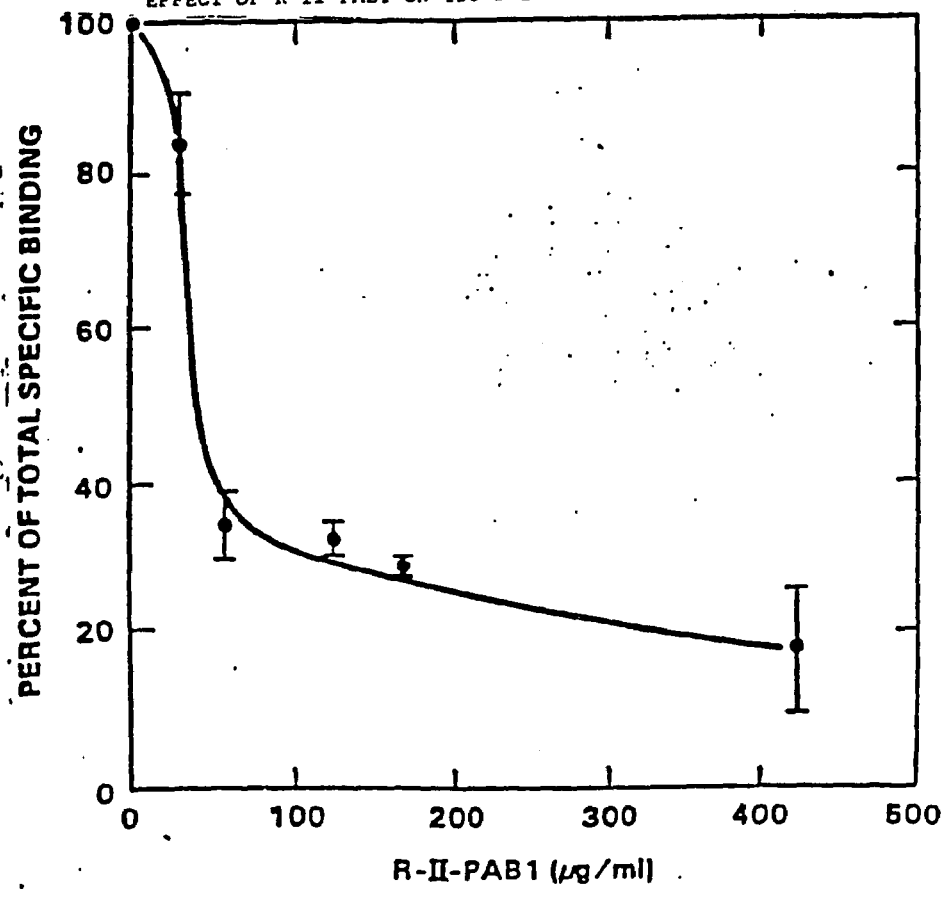


Figure 15

Effect of R-II-PAB1 on binding of 125-I-IGF-I and IGF-II to 18-54, SF cells:

Autoradiograms of 18-54, SF membranes (200ug) cross-linked to 125-I-IGF-I (lanes 1-5), or 125-I-IGF-II (lanes 6-10). 6% separatig gels were run in SDS under reducing conditions (0.1 M dithiothreitol + 5% v/v 2-mercaptoethanol). Cross-linking was performed in the presence of buffer (lanes 1 and 6); excess partially purified 500ng/ml IGF-I (lane 2); 200ng/ml IGF-II (lane 7); insulin 100ug/ml (lane 3 and 8) Pab: polyclonal antibody R-II-PAB1, 100ug/ml (lanes 4 and 9)PI: preimmune serum, 100ug/ml IgG (lanes 5 and 10).

Fig 15

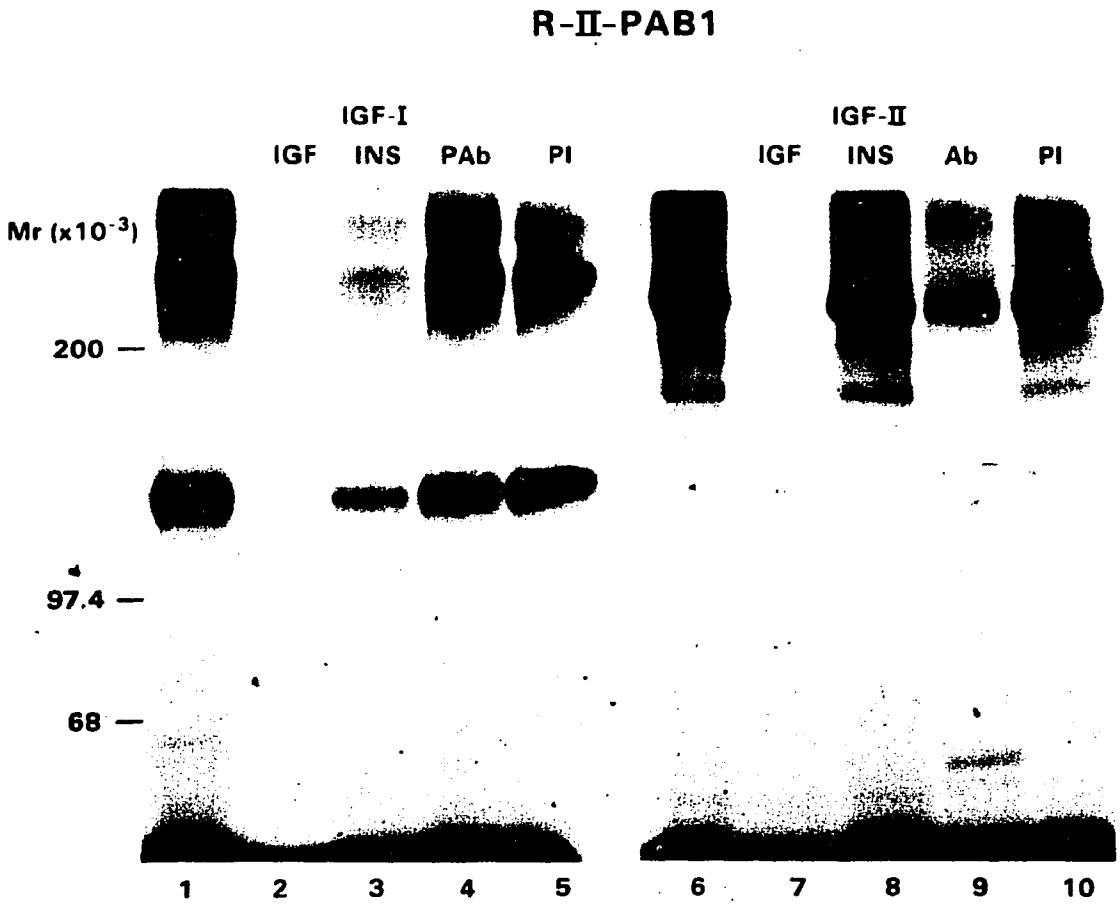

R-II-PAB1
IGF-I
IGF-II
IGF
INS
PAb
PI
IGF
INS
Ab
PI
Mr (x10-3)
200
97.4
68
1
2
3
4
5
6
7
8
9
10

Figure 16

Effect of R-II-PAB1 on replication of 18-54, SF cells.

Cells were incubated for 5 days in serum free Coon's modified Ham's F-12 medium in the presence of the indicated concentrations of R-II-PAB1 (●). Additionally, one set of dishes was incubated with 10 ng/ml rIGF-II (△), while a second set of dishes was incubated with 10ng/ml rIGF-II plus 36ug/ml R-II-PAB1 (O). Each point represents the mean + S.D. cell count of triplicate dishes counted on day 5.

B. Effect of antibody on cell number.

Fig 16(b)
EFFECT OF R-II-PAB1 ON REPLICATION OF 18-54, SF CELLS

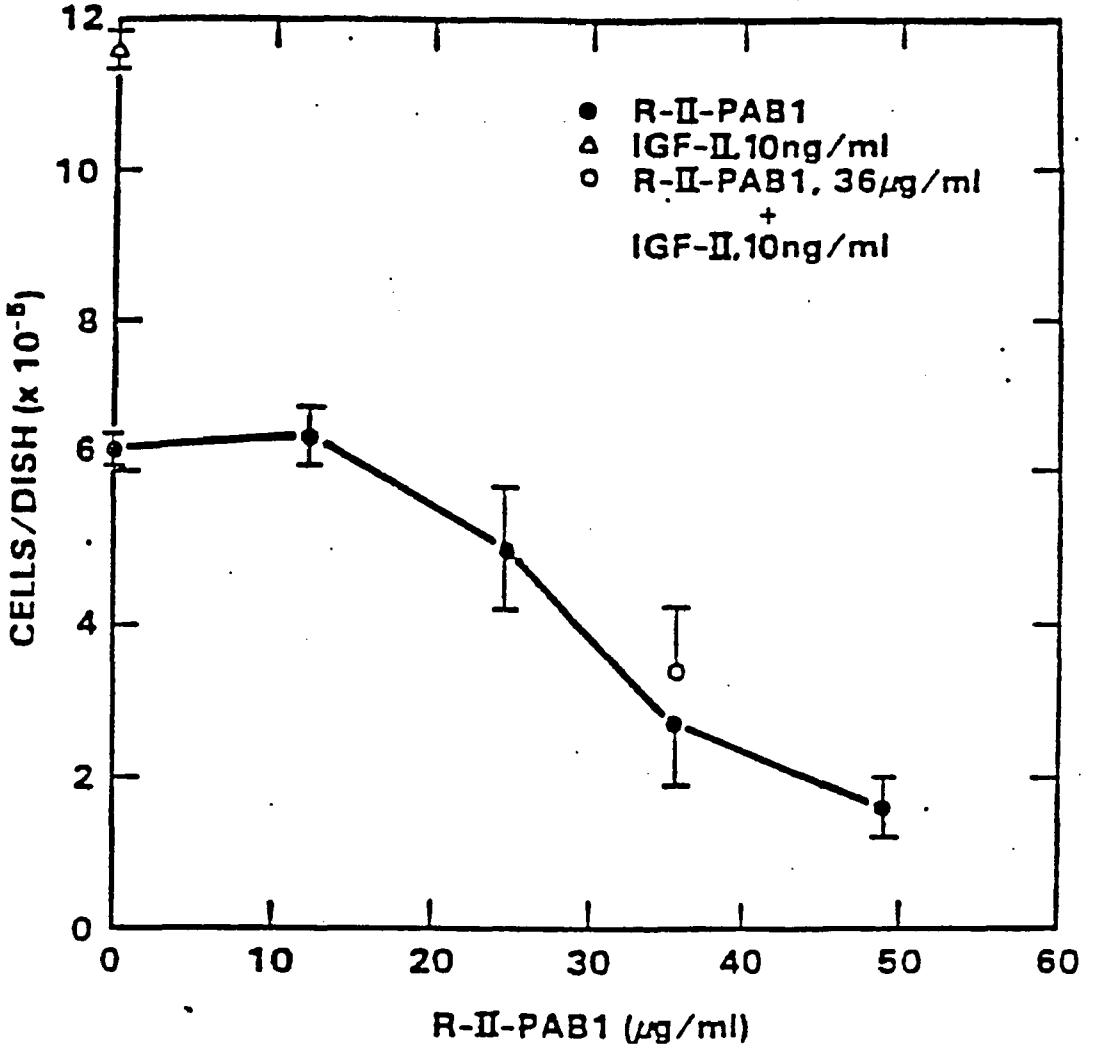


Figure 17

Effect of R-II-PAB1 on replication of 18-54,SF Cells.

Cells were incubated for 5 days in serum-free Coon's modified Ham's F-12 medium in the presence of the indicated concentrations of R-II-PAB1.

FIG 17

18-54, SF: INHIBITION OF CELL REPLICATION

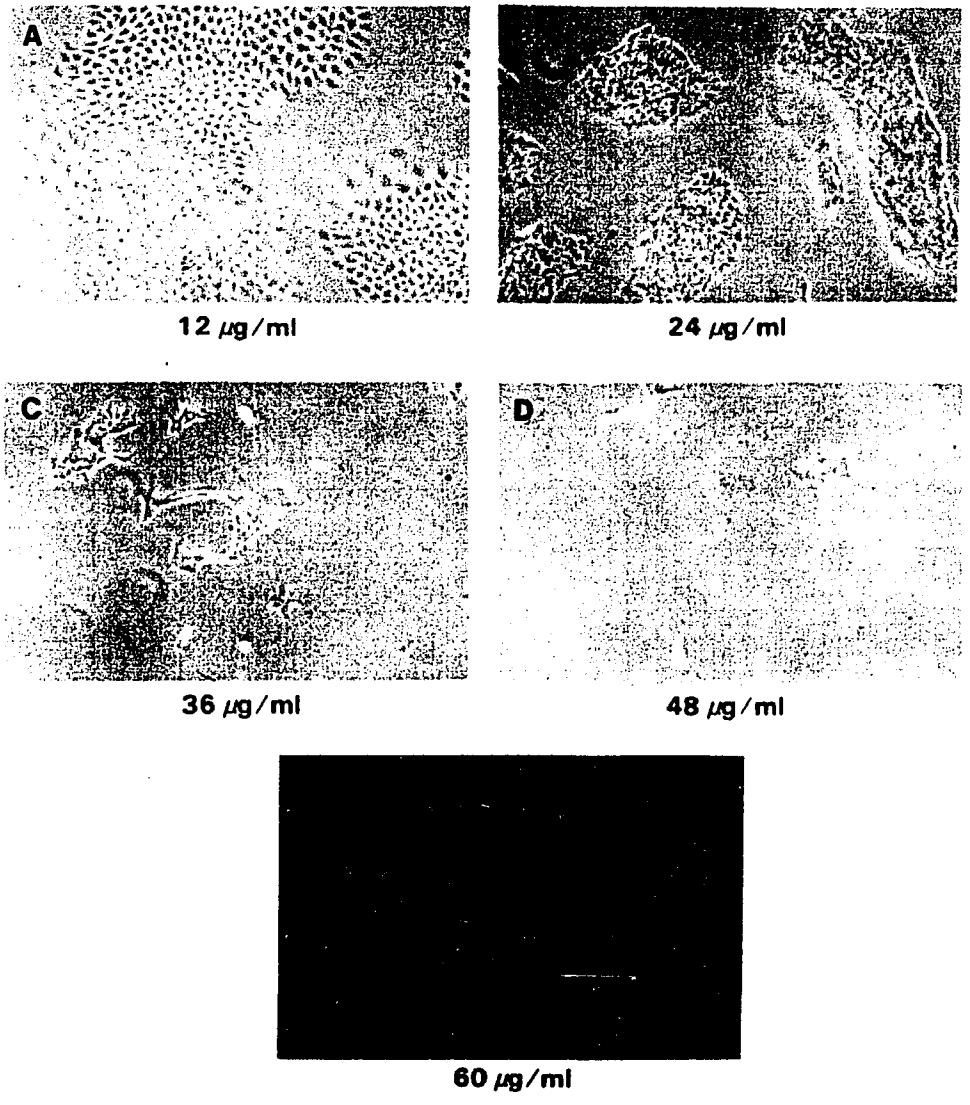


Figure 18

Effect of R-II-PAB1 on sparsely plated 18-54,SF Cells.

Cells were cultured in the presence of PBS (control), and IgG from preimmune serum (PI 100ug/ml) added at either 0 or 24 hours, or R-II-PAB1 (100ug/ml) at either 0 or 24 hours. At the end of 7 days, cells were stained with 0.5% methylene blue in 70% isopropanol. After counting the number of colonies per well, the number of cells per colony was determined by averaging the cell counts of three representative colonies in each well. Bars represent the mean + S.D. counts in triplicate wells.

Fig 18

EFFECT OF R-II-PAB1 ON SPARSELY PLATED CELLS

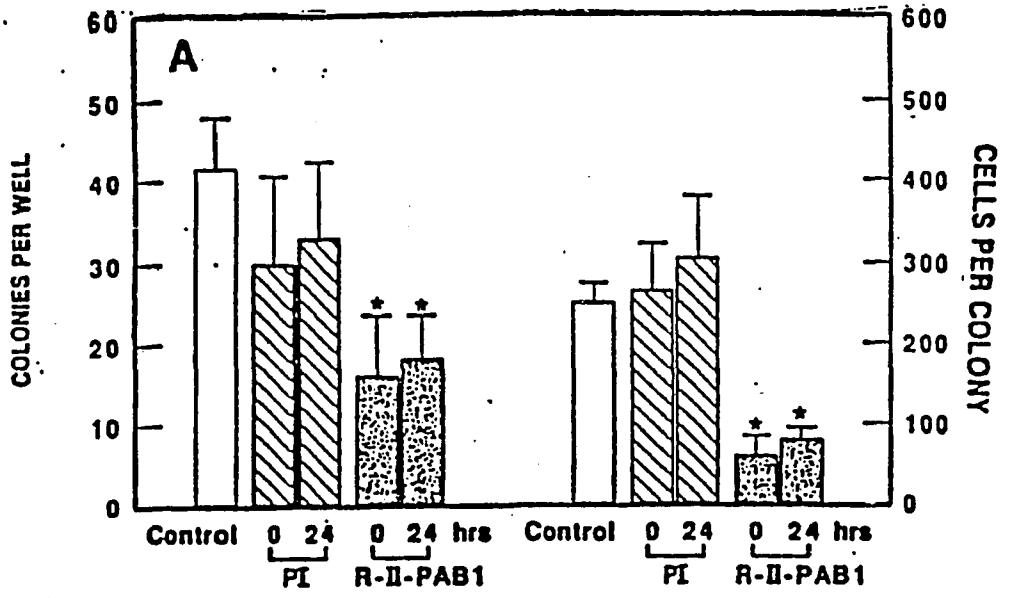


Figure 19

Effect of R-II-PAB1 on Sparsely Plated 18-54, SF cells.

Cells were cultured in the presence of PBS (control). IgG from preimmune serum (PI 100 ug/ml) or R-II-PAB-I (100 ug/ml), was added a 24 hrs., as described in Fig 18. The total number of cells per well was the product of (number of colonies per well) x (number of cells per colony).

Fig 19 Total number of cells per well

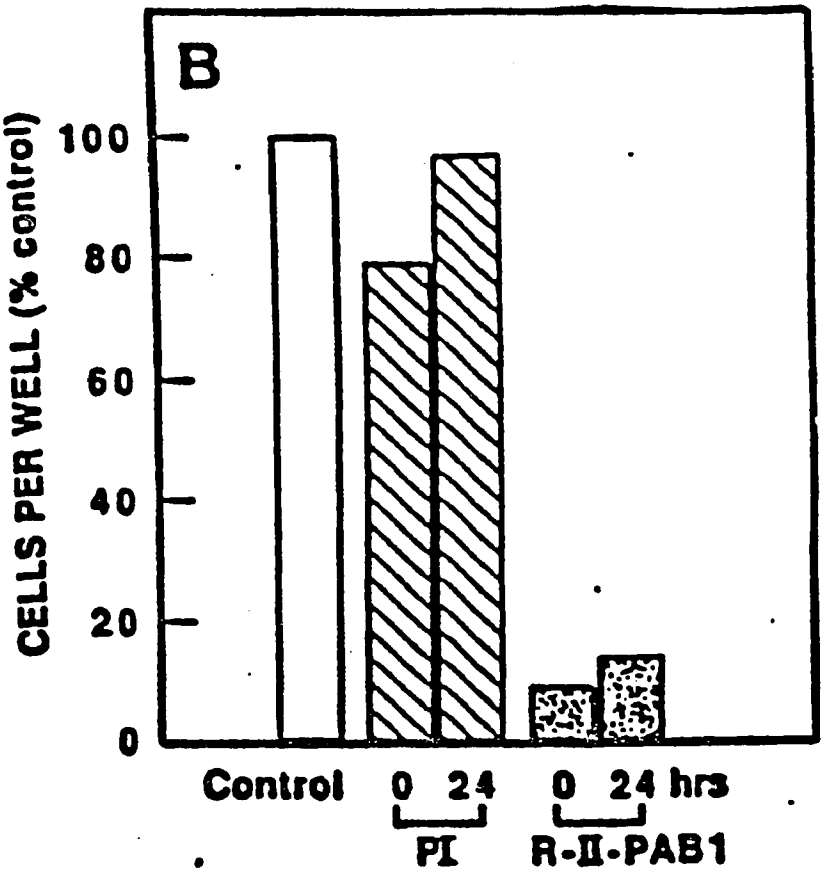


Figure 20

Approximately 100 cells were plated on single cells in 4 cm diameter plastic wells in F-12 medium supplemented with 0.5% fetal bovine serum, and either R-II-PAB1 (100ug/ml) or control IgG. After 5 days cell colonies were stained with 0.5% methylene in 70% isopropanol. Each spot represents an individual cell colony.

FIG 20 **18-54, SF: INHIBITION OF CELL REPLICATION**

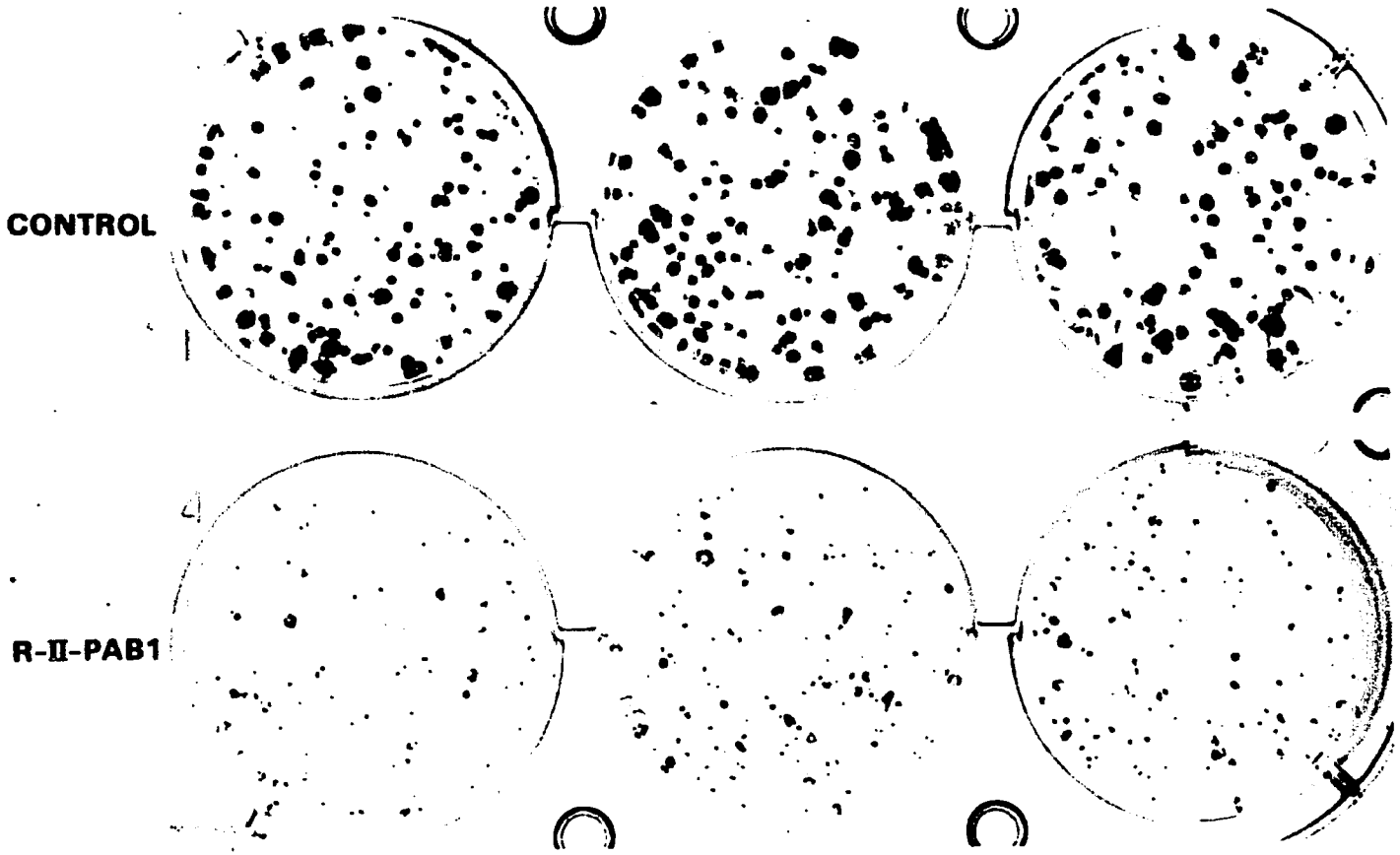


Figure 21

Morphology of individual colonies.

FIG 21

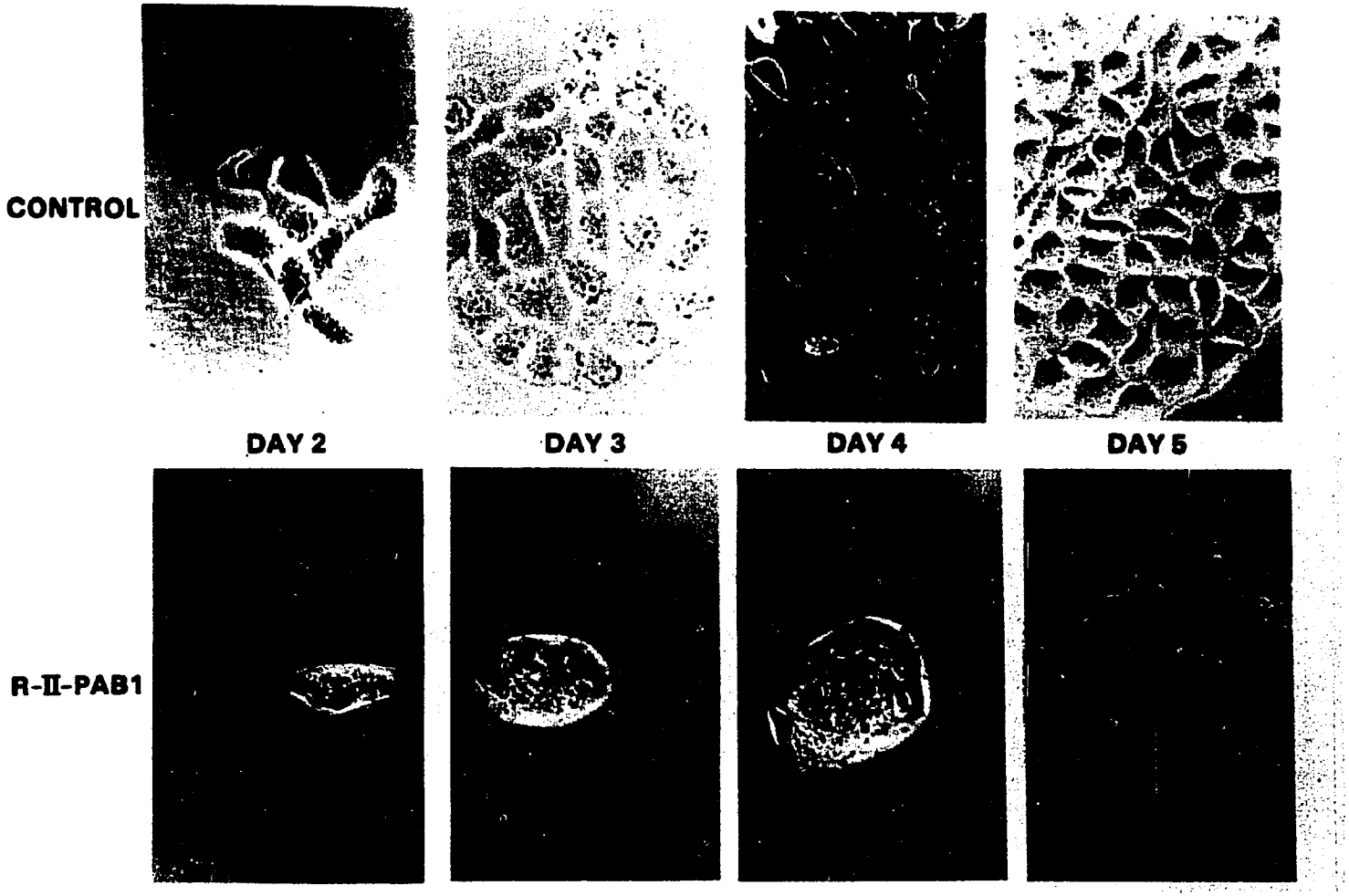


Figure 22

Schematic comparison of IGF-II receptor with receptors of related compounds. CD-M6P = cation dependant mannose-6-phosphate, NGF= nerve growth factor, LDL = low density lipoprotein . All proteins are drawn to the same scale. Extracellular cysteine-rich regions and repeat sequences are indicated by boxes. The dark band represents the type-II fibronectin homology region. The tyrosine kinase domains of insulin and IGF-I receptors are shown by boxes containing diagonal lines.

(Taken from Morgan et al., 1988).

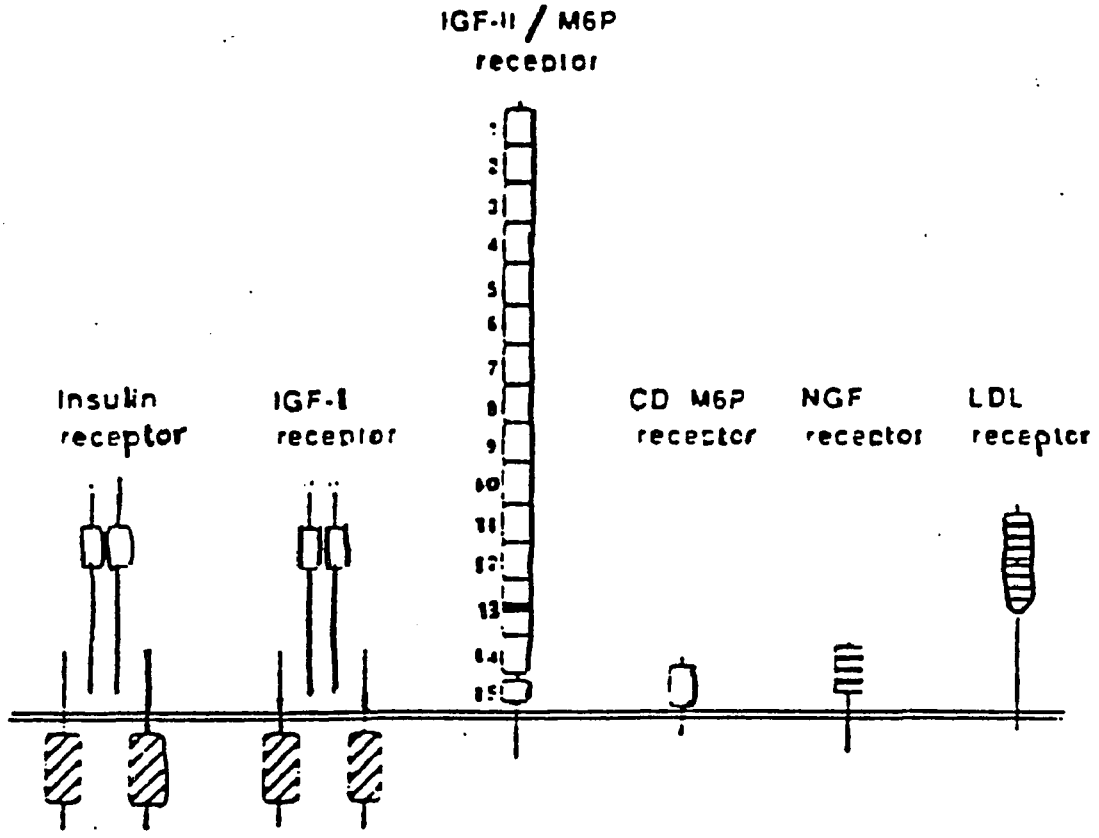

IGF-II / M6P
receptor
Insulin
receptor
IGF-I
receptor
CD M6P
receptor
NGF
receptor
LDL
receptor

Fig 22

Figure 23

Structure and expression of the IGF-II gene.

The organization of the genes encoding human and rat IGF-II is depicted. Exons are numbered 1 through 8 for human IGF-II, and I through 6 for rat IGF-II. Alternative promotors are designated with the letter P. Indicated below each gene are the mRNAs that result from transcription using different promotors. The coding regions of the mRNAs are marked by AUG at th beginning and UGA at the end. Possible alternative polyadenylation sites are noted by unlabeled arrows.

(Taken from Daughaday and Rotwein 1989)

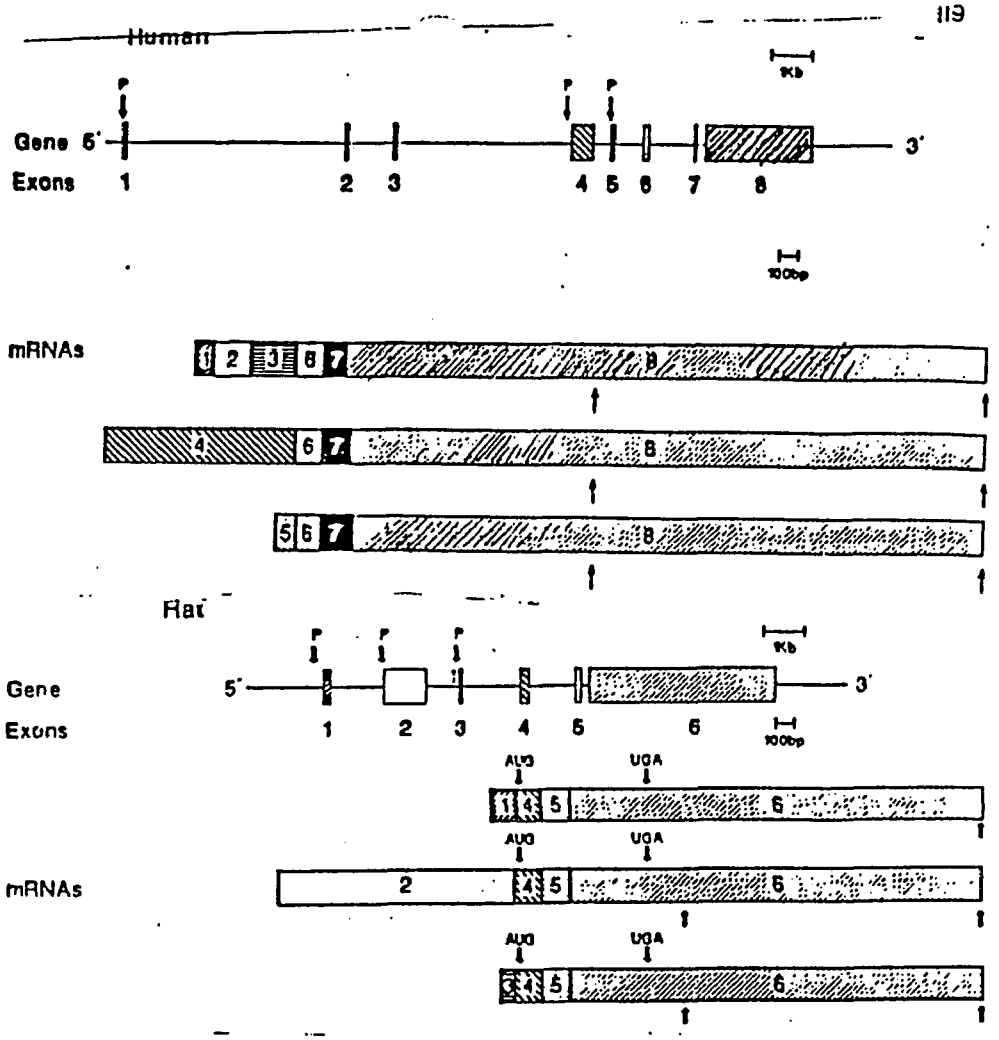


FIG. 23 Structure and expression of the IGF-II gene. The organization of the genes encoding human and rat IGF-II is depicted. Exons are numbered 1 through 8 for human IGF-II and 1 through 6 for rat IGF-II. Alternative promoters are designated with the letter P. Indicated *below each gene* are the mRNAs that result from transcription using the different promoters. The coding regions of the mRNAs are marked by AUG at the beginning and UGA at the end. Possible alternative polyadenylation sites are noted by *unlabeled arrows*.

Figure 24

Nucleotide sequences of 5'-untranslated regions of rat IGF-II, E1, E2, and E3 with the common connecting 5' extremity of the coding exon E4. The ATG initiator is underlined.

(Taken from Ueno T. et al., 1988)

FIG 24

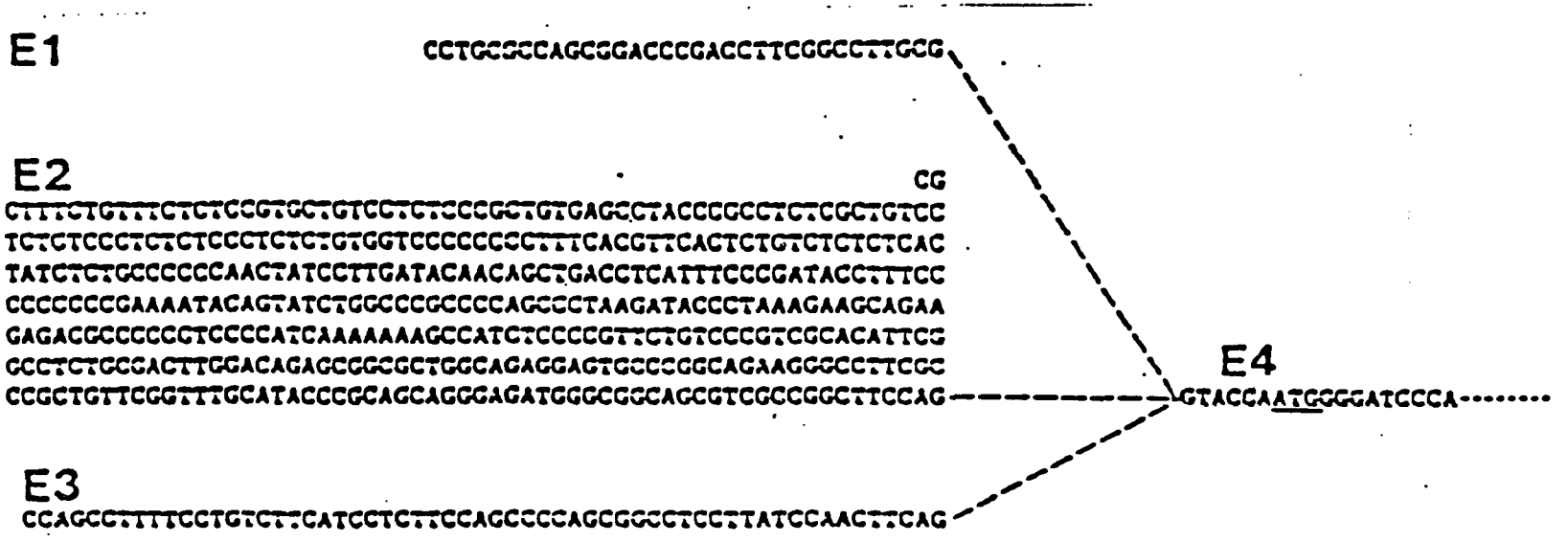

E1
CCTGCGCCAGCGGACCCGACCTTCGGCCTTGCG
E2
CG
CTTTCTGTTTCTCTCCGTGCTGTCCTCTCCCGCTGTGAGCCTACCCGCCTCTCGCTGTCC
TCTCTCCCTCTCTCCCTCTCTGTGGTCCCCCCCCTTTCACGTTCACTCTGTCTCTCTCAC
TATCTCTGCCCCCCAACTATCCTTGATACAACAGCTGACCTCATTTCCCGATACCTTTCC
CCCCCCCGAAAATACAGTATCTGGCCCGCCCCAGCCCTAAGATACCCTAAAGAAGCAGAA
GAGACGCCCCCGTCCCCATCAAAAAAAGCCATCTCCCCGTTCTGTCCCGTCGCACATTCG
GCCTCTGCGACTTGGACAGAGCGGCGCTGGCAGAGGAGTGCCCGGCAGAAGGGCCTTCGC
CCGCTGTTCGGTTTGCATACCCGCAGCAGGGAGATGGGCGGCAGCGTCGCCGGCTTCCAG
E4
GTACCAATGGGGATCCCA
E3
CCAGCCTTTTCCTGTCTTCATCCTCTTCCAGCCCCAGCGGCGTCCTTATCCAACTTCAG

Figure 25

Southern analysis of clone 22 for restriction mapping.

DNA was extracted from the clone (22) obtained on screening the genomic library from the 18-54, SF cells. It was digested with restriction enzymes Bg(BglII), H(HindIII), S1(SacI), XB (XbaI), XO (XhoI), R(EcoRI). The line indicated by R shows lanes double digested with EcoRI plus the indicated restriction enzyme eg P (PstI), Pv(PvuII). The unmarked lanes are the molecular weight markers: DNA restricted with HindIII.

DNA was electrophoresed on a 1% agarose gel, transferred on to nitrocellulose, and hybridized with the 545bp cDNA probe as described in Materials and Methods.

FIG 25
SOUTHERN BLOT FOR RESTRICTION ANALYSIS

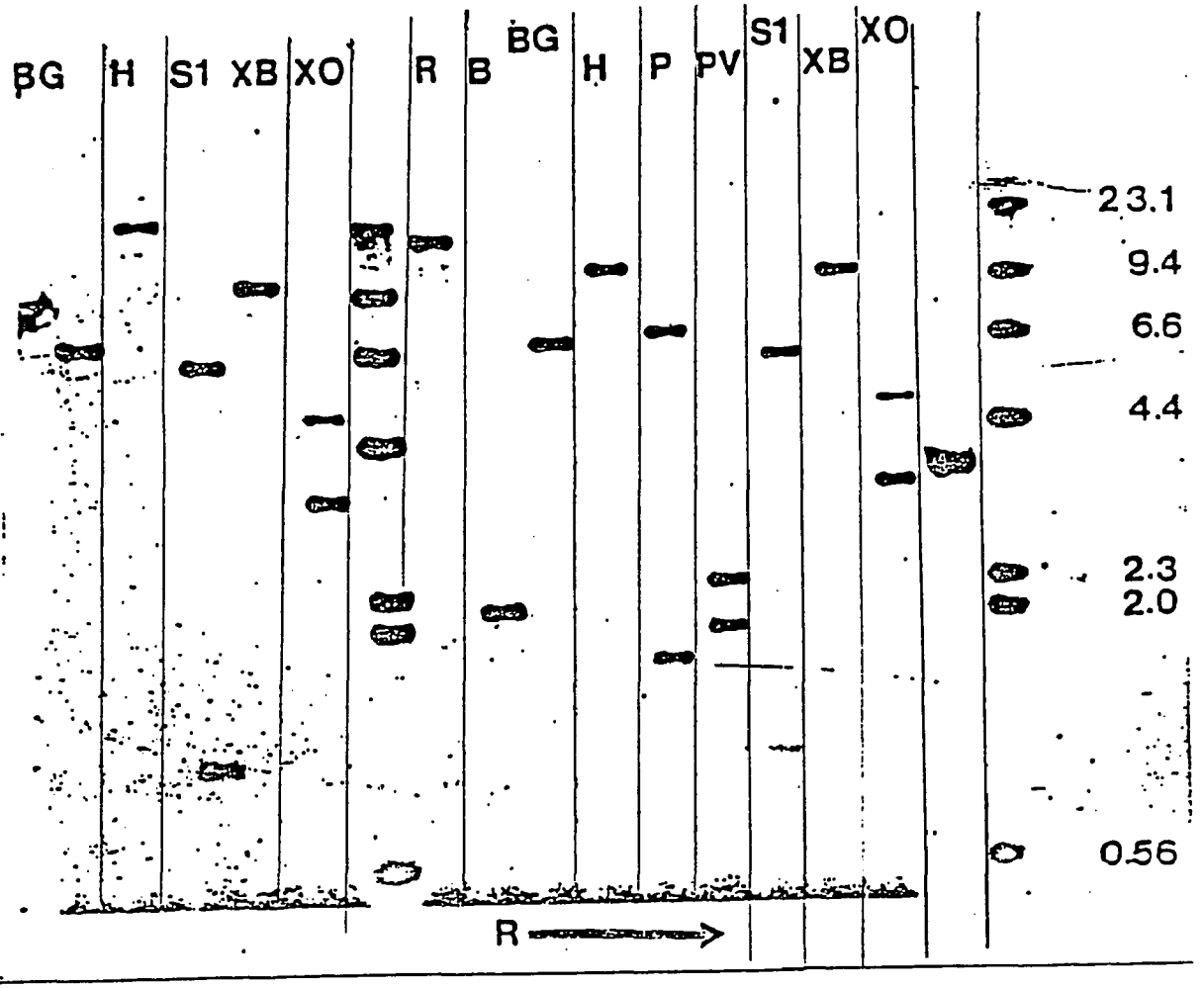


Figure 26

A. Detailed restiction map of the rIGF-II clone(22).

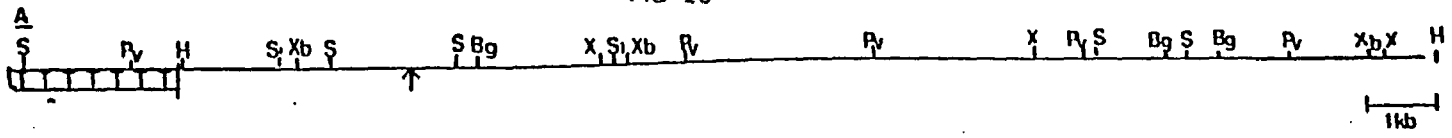
S = SacI, Pv = PvuII, H = HindIII, S = SalI, Xb = XbaI, Bg = BglII. The 5' region that has not been studied so far is highlighted by an open box.

B. The sizes of the various restriction fragments obtained from which the final restriction map was derived.

Figure 26(a)
Structural organization of rIGF-II gene.

The top line indicates the restriction map of the rIGF-II genomic clone. The restriction sites to subclone various fragments are indicated. B = BamHI; Bg = BglII; R1 = EcoRI; R5 = EcoRV; H = HindIII; K = KpnI; P = PstI; Pv = PvuII; S1 = SacI; S2 = SacII; S = StuI;X = XhoI.

FIG 26


A
S
Pv
H
S
Xb
S
S
Bg
X
S
Xb
Pv
Pv
X
Pv
S
Bg
S
Bg
Pv
Xb
X
H
1kb

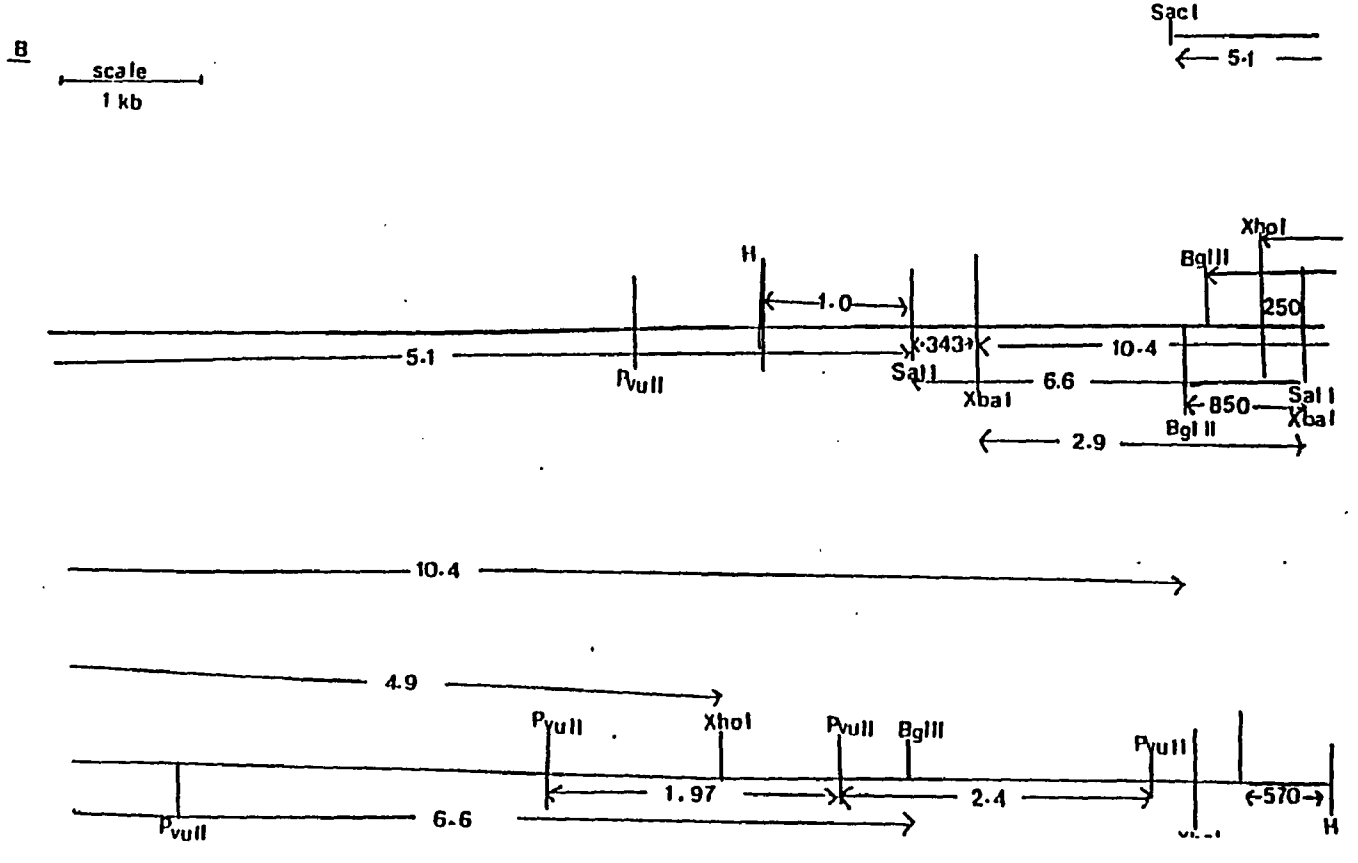

B
scale
1 kb
SacI
5.1
H
1.0
Xhol
BglII
250
5.1
PvuII
.343
SalI
10.4
Xbal
6.6
850
SalI
Xbal
BglII
2.9
10.4
4.9
PvuII
XhoI
PvuII
BglII
PvuII
1.97
2.4
570
PvuII
6.6
H

Figure 27

Sequence analysis of the SalI-SalI fragment cloned in M13.

```
        10          20          30          40          50          60
     *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
ATCCCCCCAA ATCCTAGGTT TTHMATATCT GTGTCTCACA AATTTCCTAG CTACTAATTC
TAGGGGGGTT TAGGATCCAA AADKTATAGA CACAGAGTGT TTAAAGGATC GATGATTAAG
_________10a____DM25T3I________30a_____>

        70          80          90          100         110         120
     *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
TGTTCCCTGT AATTTAGATT TCAACATCCA TTGGGTAATT TTTATTGATC CCCAAAACAG
ACAAGGGACA TTAAATCTAA AGTTGTAGGT AACCCATTAA AAATAACTAG GGGTTTTGTC

        130         140         150         160         170         180
     *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
GGAGTATGTT TCTCTAAGAA CAAACATTGT CCAAGAAAAC ATACCCCCCT TGTTTAACAA
CCTCATACAA AGAGATTCTT GTTTGTAACA GGTTCTTTTG TATGGGGGGA ACAAATTGTT

        190         200         210         220         230         240
     *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
AAAGATCAAT AGGAATCTGT TTAAGAAATT AAAATATTAA AAGGCATTGA TTTTTCTGTC
TTTCTAGTTA TCCTTAGACA AATTCTTTAA TTTTATAATT TTCCGTAACT AAAAAGACAG

        250         260         270         280         290         300
     *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CTTCTCAAAT GGACTGATTA TACTCCTAAA GGCCCCACAT TTAGACAGCA TTTAGCCCAC
GAAGAGTTTA CCTGACTAAT ATGAGGATTT CCGGGGTGTA AATCTGTCGT AAATCGGGTG

        310         320         330         340         350         360
     *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
TCTCTCTCTA GAGGACTTCT CTGAAGCCAC AGAAATTAGA GGTGACTTTT ACCCGGGTGC
AGAGAGAGAT CTCCTGAAGA GACTTCGGTG TCTTTAATCT CCACTGAAAA TGGGCCCACG
     309 XbaI
```

Figure 28

Southern Blot of total genomic DNA from 18-54, SF cells, adult rat liver cells and BRL-3A cells.

Genomic DNA from the 18-54,SF cells , adult rat liver cells and BRL-3A cells were digested Bam HI (B), BglII (Bg), HindIII (H), PvuII, SacI (S1), SacII(S2), XbaI(Xb) and Xho(XO). The DNA was run on a 1% agarose gel , transferred to nitrocellulose, and probed with the rIGF-II cDNA probe.

Fig 28.
SOUTHERN BLOT OF GENOMIC DNA CUT WITH DIFFERENT ENZYMES

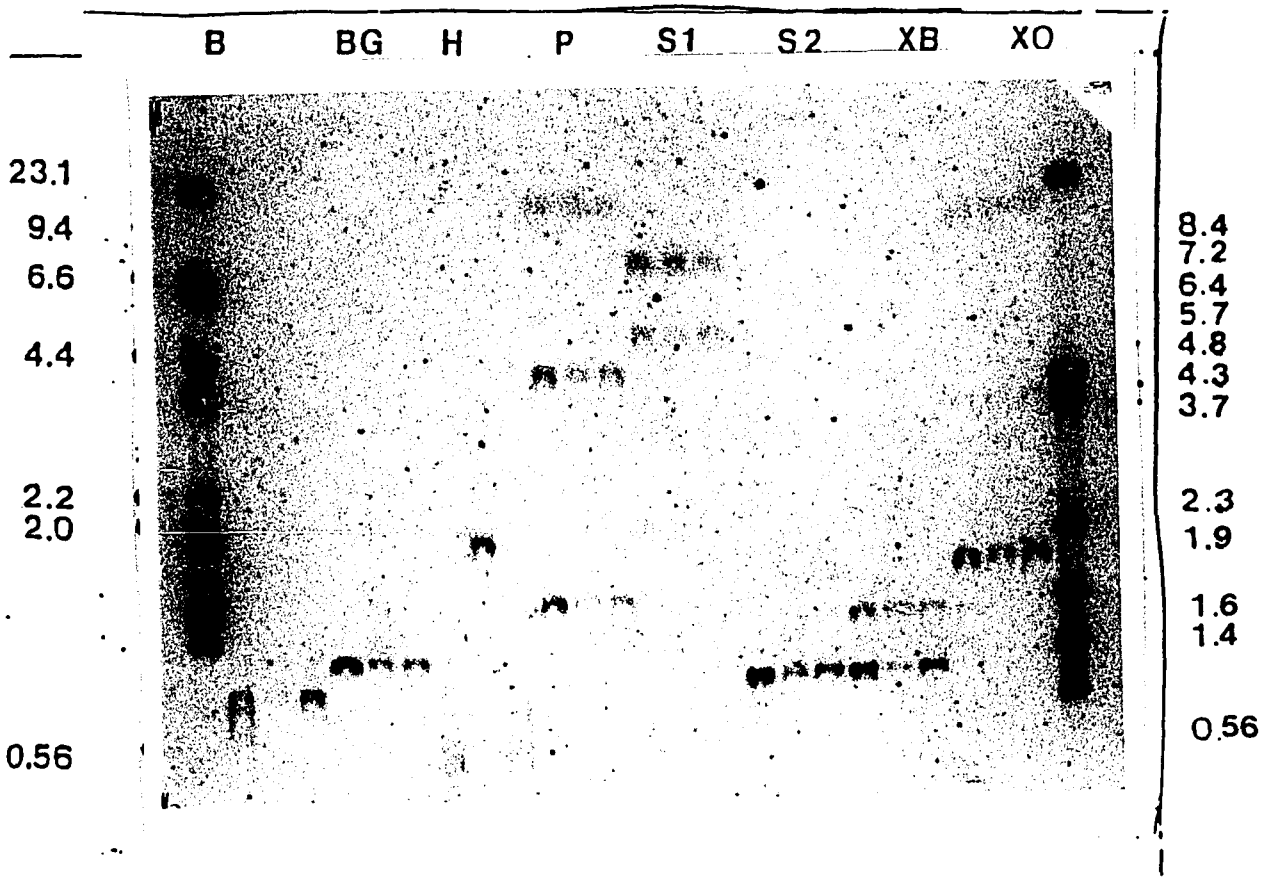


Table 1. Comparison of Insulin and IGF Receptors

	Insulin	Type I IGF	Type II IGF
M_r (unreduced)	>300,000	>300,000	240,000
M_r (reduced)	135,000 90,000	135,000 90,000	260,000
Subunits	2 alpha + 2 beta	2 alpha + 2 beta	none
Affinity	Ins > IGF-II > IGF-I	IGF-I > IGF-II > Ins	IGF-II >> IGF-I
Affinity for Insulin	high	low	none
Glycosylation	+	+	+
Transmembrane	+	+	probable
Tyrosine Kinase	+	+	-

Effect of various peptides on ^{125}I-insulin specific binding to 18-54,SF cell membranes

^{125}I-insulin ng/ml	Unlabeled Competitor	^{125}I-insulin mean cmp ± SEM	% Total Bound ^{125}I-insulin
1.7	None	2979 ± 72	100
"	porcine INS, 2000 ng/ml	1769 ± 56	59.4
"	human INS, " "	1916 ± 105	64.3
"	bovine INS, " "	2128 ± 113	71.4
"	ovine INS, " "	2283 ± 279	76.6
"	equine INS, " "	2448 ± 170	82.2
"	MSA, 1000 ng/ml	2172 ± 69	72.9
"	pituitary FGF, 5000 ng/ml	3013 ± 234	101.4
"	C-peptide, 2500 ng/ml	2906 ± 26	97.5
"	human PRL, 1000 ng/ml	3035 ± 66	101.9
"	Neurotensin, 5000 ng/ml	3134 ± 266	105.2
"	Substance P, " "	3377 ± 81	113.4
"	Bombesin, " "	3372 ± 181	113.2
"	GnRH, " "	3503 ± 280	117.6
"	TRH, " "	3206 ± 184	107.6
"	EGF, " "	2848 ± 92	95.6
"	7sNGF, 1000 ng/ml	2991 ± 344	100.4

Table II

|| Cell membranes were prepared as outlined in Materials and Methods. One hundred micrograms of membrane protein were added to assay tubes containing 1.7 ng/ml ^{125}I-insulin (INS) without or with various peptide competitors., Tubes were incubated for 90 min. at 24°C, centrifuged at 13,700 xg for 3 min. and the supernatant removed. The tubes containing the membrane pellet were counted in a gamma counter. Counts per minutes are the mean from triplicate determinations from one experiment performed twice.

TABLE III. Effect of various peptides on 125-I-IGF-II specific binding in 18-54, SF cell membranes.

125-I-IGF-II ng/ml	Unlabelled competitor (1ug/ml)	125-I-IGF-II mean cpm ± SEM	%Specific bound
50 ng/ml	None	5610± 200	100
"	MSA 1ug/ml	1060 ± 53	84.7
"	IGF-II from 18s	1661 ± 80	79.3
"	IGF-II(synthetic)	1258 ± 55	77.5
"	e Insulin	6224 ± 300	0
"	p Insulin	6458 ± 30	0
"	chain A ins	5358 ± 67	4.49
"	chain B ins	6131 ± 203	0
"	EGF 10 ug/ml	5420 ± 74	3.38
"	h growth factor	6940 ± 323	0
"	IGF-I	4043 ± 93	27.8

Table III. Cell membranes were prepared as outlined in materials and methods. 1500 ug/ml membrane protein were added to assay tubes containing 50ng/ml 125-I-IGF-II with or without various peptide competitors. Tubes were incubated at RT (25 C)for 2 hours, centrifuged at 13,200 X g for 3 mins, and the supernatant removed. the tubes containing the membrane pellet were counted in a gamma counter. Counts per minute are the mean from triplicate determinations from one experiment performed twice.E= equine, P=porcine, MSA =multiplication stimulating activity (rIGF-II).

TABLE IV. rIGF-II Production by 18-54,SF Cells

DAY	DNA (ug/ml)	IGF-II (ng/ml)
0	1.14 + 0.09	0
2	1.14 + 0.12	0
4	3.91 + 0.46	10.6 + 9.7
6	19.92 + 1.01	55.7 + 4.3
8	17.7 + 0.34	62.2 + 11.8
10	15.86 + 1.15	68.8 + 10.2

Table IV. 2 x 10 cells were grown in 60mm x 10mm dishes. Conditioned media from 3 different dishes was removed on the days indicated, and IGF-II was measured by radioreceptor assay, while DNA was measured by a flourometric method.

BIBLIOGRAPHY

Adams S.O., Nissley S.P., Handwarger S., Rechler M.M. (1983). Development patterns of insulin-like growth factor-I and II synthesis and regulation in rat fibroblasts. Nature 302. 150

Ashton I.K., Zapf J., Eischenk I.,Mackenzie I.Z. 1985. Insulin-like growth factors IGF-1 and IGF-2 in human fetal plasma, and relationship to gestational age and fetal size during mid-pregnancy. Acta Endocrinol (Copenh) 110. 558.

Beguinot F., Kahn C.R.,Moses A.C., Smith R.J. 1985. Distinct biologically active receptors for insulin, insulin-like growth factor-I and insulin-like growth factor-II in cultured skeletal muscle cells. J. Biol. Chem. 260. 15892.

Bell G.I., Stempien M.M., Fong N.M., Rall L. B. 1986. Sequences of liver cDNAs encoding two different mouse insulin like growth factor I precursors. Nuc Acids Res 14. 7873.

Blundell T.L and Humbel R.E. 1980. Homologous families: pancreatic hormones and homologous growth factors. Nature 287. 781.

Blundell T.L., Bedarkar S., Humbel R.E. 1983. Tertiary structures receptor binding, and antigenicity of insulin-like growth factors. Fed. Proc. 42. 2592.

Bradshaw R.A., Niall H.D. 1978 . Insulin related growth factors. Trends Biochem Sci 3. 274

Brissenden J.E., Ullrich A., Francke U. 1984. Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor. Nature. 310. 780.

Brown A.L., Graham D.E., Nissley S.P., Hill D.J., Strain A.J., Rechler M.M. 1986. Developmental regulation of IGF-II mRNA in different rat tissues. J. Biol. Chem. 261. 13144.

Chiarrotti L, Brown A.L., Frunzio R., Clemmons D.R., Rechler M.M., Bruni C.B. 1988. Structure of the rat insulin-like growth factor-II transcriptional unit: Heterogenous transcripts are generated from two promotors by use of multiple polyadenylation sites and differential ribonucleic acid splicing. Mol. Endo.2. 1116.

Conover C.A., Hintz R.L., Rosenfeld R.G. 1986. Comparitive effects of somatomedin C and insulin on the metabolism and growth of cultured human fibroblasts. J. Cell Physiol. 122. 133

Conover C.A., Misra P., Hintz R.L., Rosenfeld R.G.1986. Effect of an anti-insulin-like growth factor-I receptor antibody on insulin-like growth factor-II stimulation of DNA synthesis in human fibroblasts. Biochem Biophys Res Commun. 139. 501.

Corvera S., Whitehead R.E., Mottola C., Czech M.P. 1986. The insulin-like growth factor-II is phosphorylated by a tyrosinekinase in adipocyte plasme membrane. J. Biol. Chem. 261. 7675

Daughaday W.H., Rotwein P. 1989. Insulin-like growth factors I and II. Peptide messenger ribonucleic acid and gene structures, serum and tissue concentrations. 1989. Endocrine Reviews. 10. 68.

Daughaday W.H., Hall K., Salmon W.D., Van den Brande J.L., Van Wyk J.J. 1987. On the nomenclature of the somatomedins and insulin like growth factors. J. Clin. Endocrinol. Metab. 65. 1075.

Daughaday W.H., Mariz I.K., Trivedi B. 1981. A preferential binding site for insulin-like growth factor II in human and rat placental membranes. J.Clin.Endocrinol.Metab. 53. 282.

Dull T.J., Gray A., Hayflick J.S., Ullrich A. 1984. Insulin like growth factor II precursor gene organization in relation to insulin gene family. Nature. 310. 777.

Ellenberg M., Rifkin H. Eds. 1983 Diabetes Mellitus Theory and Practice. Medical Examination NY.

Enberg G., Carlquist M., Jornvall H., Hall K. 1984. The characterization of somatomedin A, isolated by micro computer controlled chromatography, reveals an identity to IGF-I. Eur. J. Biochem. 143. 117.

Ewton D.Z., Falen S.L., Florini J.R. 1987. The Type-II insulin-like growth factor receptor has low affinity for IGF-I analogs: Pleiotypic actions of IGFs on myoblasts are apparently mediated by the Type I receptor. Endocrinology 120. 115

Feizi T., Childs R.A. 1987. Carbohydrates as antigenic determinants of glycoprotein. Biochem J. 245. 1.

Froesh E.R., Schmid C., Schwander J., Zapf J. 1985. Actions of insulin like growth factors. Annu. Rev. Physiol. 47. 443.

Frunzio R., Chiariotti L., Brown A.L., Graham D.E., Rechler M. M., Bruni C.B. 1986. Structure and expression of the rat insulin-like growth factor gene. J. Biol. Chem. 261. 36. 17138.

Flier J.S., Kahn C.R., Roth R., Bar R.S. 1975. Antibodies that impair insulin receptor binding in an unusual diabetic syndrome with severe insulin resistance. Science 190. 63.

Furman T.C., Epp J., Hsuing H.M., Hoskins J., Long G., Mendelsohn L., Schoner B., Smith D.P., Smith M.C. 1987. Recombinant human insulin like growth factor II expressed in E. coli. Biotechnology 5. 1045

Goodyer C.G., De Stephano L., Lai W.H., Guyda H.J. Possner B.I. 1984. Characterisation of insulin like growth factor receptors in rat anterior pituitary, hypothalamus and brain. Endocrinology 114. 1187.

Graham D.E., Whitfield H.J., Nissley S.P., Seelig S., Berry S. 1986. Coordinate developmental regulation of high and low molecular weight mRNAs of rat insulin-like growth factor-II.

Proc Natl Acad Sci USA 83. 4519.

Gray A., Tam A.W., Dull T.J., Hayflick J., Pintar J., Cavenee W.K., Koufus A., Ullrich A. 1987. Tissue specific and developmentally regulated transcription of the insulin like growth factor 2 gene. DNA. 6. 283.

Grossberger D. 1987. Miniprep of DNA from bacteriophage lambda. Nuc. Acid Res. 15. 6737.

Han V.K.M., Lund P.K., Lee D.C., D.Ercole A.J. 1988. Expression of Somatomedin /insulin like growth factor messenger ribonucleic acids in the human fetus: identification , characterization, and tissue distribution. J. Clin. Endocrinol. Metab. 66. 422.

Hari J., Pierce S.B., Morgan D.O., Sara V., Smith M.C., Roth R.A. 1987 The receptor for insulin-like growth factor-II mediates an insulin like response. Biochim Biophys Acta. 988.

Haskell J.F., Nissley S.P., Rechler M.M., Sasaki N.,Greenstein L., Lee L. 1985. Evidence for the phosphorylation of Type II insulin-like growth factor receptor in cultured cells. Biochem. Biophys. Res Commun. 130. 793.

Heaton J.H., Krett N.L., Alvarez J.M., Gelehter T.D., Romanus J.A., Rechler M.M. 1984. Insulin regulation of insulin-like growth factor action in rat hepatoma cells. J. Biol. Chem. 259. 2396.

Heaton J.H., Schilling E.E., Gelehrter T.D., Rechler M.M., Spencer C., Nissley S.P. 1980. Induction of tyrosine aminotransferase and amino acid transport in rat hepatoma cells by insulin and insulin-like growth factor, multiplication stimulating activity. Biochim et Biophys Acta. 632. 192.

Honegger A., Humbel R.E. 1986. Insulin like growth factors I and II in fetal and adult bovine serum.. Purification, primary structure and immunological cross reactivity. J. Biol. Chem. 261. 569.

Hoppener J.W.M., Steenberg P.H., Slebos R.J.C. de Pachter-Holthizen P., Roos B.A., Jansen M., van den Brande J.L., Sussenbach J.S., Jansz H.S., Lips C.J.M. 1987. Expression of insulin like growth factor I and II genesin rat medullary thyroid carcinoma. FEBS Lett 215. 122.

Isaakson O.G.P., Jansson J.V., Gause I.A.M. 1982. Growth hormone stimulates longitudinal growth directly. Science. 216. 1237.

Jacobs S., Cook S., Svoboda M., Van Wyck J.J. 1986 Interaction of monoclonal antibodies IR-1 and IR-3 with insulin and somatomedim-C receptors. Endocrinology. 118. 223.

Jacobs S., Kull F.C., Cuatrecasas P. 1983. Monensin blocks the maturation of receptors for insulin and Somatomedin C: identification of receptor precursors. Proc Natl Acad Sci USA. 80. 1228.

Janeczko R.A., Etlinger J.D. 1984. Inhibition of intracellular proteolysis in muscle cultures by multiplication stimulating activity. J. Biol. Chem. 259. 6292.

Jonas H.A., Harrison L.C. 1985. The human placenta contains two distinct binding and immunoreactive species of IGF-I receptors. J. Biol. Chem. 260. 2288.

Jonas H.A., Newman J.D., Harrison L.C. 1986. An atypical insulin receptor with high affinity for insulin like growth factors copurified with placental insulin receptors. Proc. Natl. Acad. Sci. USA. 83. 4124.

Kasuga M., Sasaki N., Kahn C.R., Nissley S.P., Rechler M.M.,1983. Demonstration of two subtypes of insulin like growth factor receptors by affinity cross linking. J. Biol Chem. 256. 5306

Kiess W., Haskell J., Lee L.; Greenstein L.A., Miller B.E., Aarons L.A., Rechler M.M., Nissley P. 1987. An antibody that blocks insulin-like growth factor binding to the type-II IGF receptor is neither an agonist nor an inhibitor of IGF

stimulated biological responses in L6 Myoblasts. J. Biol. Chem. 262. 12745

Kasuga M., Van Obberghen E., Nissley S.P., Rechler M.M. 1981. Demonstration of two subtypes of insulin like growth factor receptors by affinity cross linking. J. Biol. Chem 256. 5305.

King G.L., Kahn C.R., Rechler M.M., Nissley S.P. 1986. Direct demonstration of seperate receptors for growth and metabolic activity of insulin and MSA using antibodies to the insulin receptor. J. Clin Invest. 66. 130.

King G.L., Kahn C.R., Rechler M.M., Nissley S.P., 1980. Demonstration of seperate receptors for growth and metabolic activity of insulin and multiplication stimulating activity (an insulin-like growth factor) using antibodies to the insulin receptor. J. Clin. Invest. 66. 130.

Klapper D.G., Svoboda M.E., Van Wyk J.J. 1983. Sequence analysis of somatomedin-C: conformation of identity with insulin like growth factor I. Endocrinology. 112. 2215.

Kojima I., Nishimoto I., Iiri T., Ogata E., Rosenfeld R.G.,1987. Evidence that type-II insulin like growth factor receptor is coupled to a calcium gating system in Balb/c 3T3 cells.

Koontz J.W., Iwahashi M. 1985. Insulin as a potent, specific growth factor in a rat hepatoma cell line. Science 211. 947

Krett N.L., Heaton J.H., Gelehrter T.D. 1987. Mediation of insulin-like growth factor actions by the insulin receptor in H-35 rat hepatoma cells. Endocrinology. 120. 483.

Lee P.D.K. Hodges D., Hintz R.L., Rosenfeld R.G. 1986. Identification of insulin like growth factor II receptors in pituitary cells. Biochem. Biophys. Res. Comm. 134. 595.

Lobel P., Dahms N.M., Brellmeyer J., Chirgwin J.M., Kornfeld S. 1987. Cloning of the bovine 215 kD cation independant mannose-6-phosphate receptor.. Proc. Natl. Acad. Sci. 84. 2233.

Lowry O.H., Rosebrough N.F., Farr A.L., Randall R., 1951. Protein measurement with Folin phenol reagent. J. Biol Chem. 193. 265.

Lund P.K., Moats-Staats B.M., Hynes M.A., Simmons J.G., Jansen M., D'Ercole A.J., Van Wyck J.J. 1986. Somatomedin/Insulin-like growth factorI and II mRNA in rat fetal and adult tissue. J. Biol Chem. 261. 14539.

MacDonald R.G., Pfefer S.R., Coussens L., Tepper M.A., Brocklebank C., Mole J., Anderson J.K., Chen A., Czech M.P., Ullrich A. 1988. A single receptor binds both insulin like growth factor II and mannose-6-phosphate. Science. 239. 1134.

MacDonald R.G., Czech M.P. 1985. Biosynthesis and processing of type-II insulin-like growth factor receptor in H-35 hepatoma cells. J. Biol Chem. 260. 11357.

Marquardt H., Todaro G.H., Henderson L.E., Oroszlan S. 1981. Purification and primary structure of a polypeptide with multiplication stimulating activity from rat liver cell cultures. Homology with human IGF-II. J. Biol. Chem. 256. 6859.

Massague J., Blinderman L.A., Czech M.P. 1982. The high affinity insulin receptor mediates growth stimulation in rat hepatoma cells J. Biol. Chem. 257. 13958.

Massague J., Czech M. P. 1982. The subunit structures of two distinct receptors for insulin like growth factors I and II and their relationship to the insulin receptor. J. Biol. Chem. 257. 5038.

Merimee T.J., Zapf J., Froesch E.R. 1982. Insulin-like growth factors (IGFs) in pygmies and subjects with the pygmy trait: Characterizations of the metabolic actions of IGF-I and IGF-II in man. J Clin Endocr Metab. 55. 1081.

Merimee T.J., Zapf J., Froesch E.R. 1981. Dwarfism in the pygmy. An isolated deficiency of IGF-I. N Eng J Med. 305. 965.

Mill J.M., Chao M.V., Ishii D.N. 1985. Insulin, insulin-like growth factor II and nerve growth factor effects on tubulin mRNA levels and neurite formation. Neurobiology. 82. 7126.

Morgan D.O., Edman J.C., Standring D.N., Fried V.A., Smith M.C.,Roth R.A., Rutter W.J. 1987. Insulin like growth factor II receptor as a multifunctional binding protein. Nature. 329. 301.

Morgan D.O., Roth R.A., 1987. Acute insulin action requires insulin receptor kinase activity: Introduction to an inhibitory monoclonal antibody into mammalian cell blocks the rapid effects of insulin. Proc Natl Acad Sci. USA 84. 41

Moses A.C., Nissley P., Short P.A., Rechler M.M., White R.M., Knight A.B., Higa O.Z. 1980. Elevated levels of insulin like growth factor activity in fetal rat serum. Proc Natl Acad Sci. USA. 77. 3649.

Mottola C., Czech M.P. 1984. The type-II insulin-like growth factor receptordoes not mediate increased DNA synthesis in H-35 hepatoma cells. J. Biol. Chem 259. 12705.

Mowbray S. Koshland D. 1987. Additive and independant responses in a single receptor : Aspartate and Maltose stimuli on the `tar' protein. Cell. 50. 171-180.

Nagarajan L., Anderson W.B., Nissley S.P., Rechler M.M., Jetten A.M. 1985. Production of insulin-like growth factor-II (MSA) by endoderm like cells derived from embryonal carcinoma cells: Possible mediator of embryonic cell growth. J. Cell Physiol. 124. 199.

Nishimoto I., Hata Y., Ogata E., Kojima I. 1987. Insulin-like growth factor II stimulates calcium influx in competent BALB/c 3T3 cells primed with epidermal growth factor. 202. 25. 12120.

Nishimoto I., Ohkuni Y., Ogata E., Kojima I., 1987. An autocrine/paracrine role for insulin likegrowth factors in human placental growth. Biochem Biophys Res Commun. 142. 275.

Nissley S.P., Short P.A., Rechler M.M., Podskalny J.M., Coon H. 1977. Proliferation of Buffalo rat liver cells in serum free medium does not depend upon multiplication stimulating activity. (MSA). Cell. 11. 441.

Oppenheimer C.L., Pessin J.E., Massague J., Gitomer W., Czech M.P.. 1983. Insulin action rapidly modulates the apparent affinity of the insulin-like growth factor receptor. J. Biol. Chem. 258. 4824

Pagter-Holthuizen P., Jansen M., Schaik R. Oosterwijk C., Van den Brande J.L. Sussenbach J.S.1987. The human insulin like growth factor II gene contains two development specific promotors. FEBS Letters. 214. 259-264.

Philips H.J. 1973. Dye exclusion tests for cell viability. In: use P.F., Patterson M.K. Tissue culture : Methods and Applications. New York Acedemic Press. 406.

Radeke M.J., Misko T.P., Hsu C., Herzenberg L.A., Shooter E.M. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature. London 325. 593.

Rechler M.M., Nissley S.P. 1985. The nature and regulation of the receptors for insulin like growth factors. Annu. Rev. Physiol. 47.425.

Reeve A.E., Ecoles M.R., Wilkins R.J., Bell G.I., Millow L.J. 1985. Expression of insulin-like growth factor-II transcripts in Wilm's tumor. Nature 317.

Rinderknecht E., Humbel R.E. 1978. Primary structure of insulin like growth factor II. FEBS Letters. 89. 283.

Rinderknecht E., Humbel R.E. 1976 . The aminoacid sequence of human insulin like growth factor I and its structural homology with proinsulin. J.Biol. Chem. 253. 2769.

Rosen O.M 1987. After insulin binds. Science 237. 1452

Rosenfeld R.G. Ceda G., Wilson D.M., Dollar L.A., Hoffman A.R. 1984. Characterization of high affinity receptors for insulin like growth factors I and II on rat anterior pituitary cells. Endocrinology. 114. 1571.

Rosenfeld R.G., Hodges D., Phan H., Lee P.D.K., Powell D.R. 1986. Purification of the insulin like growth factor-II receptor from an IGF-II producing cell line, and generation of an antibody which blocks immunoprecipitates and blocks the type II IGF receptor. Biochem. Biophys. Res Comm. 138. 304.

Rubin J.S., Mariz I., Jacobs J W., Daughaday W.H. Bradshaw R.A. 1983. Isolation and partial sequence analysis of rat basic somatomedin. Endocrinology. 110. 734.

Rubin J.B., Shia M.A., Pilch P.F. 1983. Stimulation of tyrosine specific phosphorylation in vitro by insulin-like growth factor I. Nature 305. 438

Salmon Jr W.D., Daughaday W.H. 1957. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J. Lab. Clin. Med 49. 825.

Sara V.R., Hall K., Von Holtz H., Humbel R., Sjogren B., Wettenberg L. 1982. Evidence of the presence of specific receptors for insulin-like growth factors-I (IGF-I) and II (IGF-II) and insulin throughout the adult human brain. Neurosci. Lett. 34. 39.

Schoenle E., Zapf J., Humbel R.E., Froech E.R. 1982. Insulin-like growth factor-I stimulates growth in hypophysectomized rats, Nature. 296. 252.

Scott J., Cowell J., Robertson M.E., Preistly C.M., Waeley R., Hopkins B., Pritchard J., Bell G.I., Rall L.B., Graham C.F., Knott T.J. 1985. Insulin-like growth factor II gene

expression in Wilm's tumor and embryonic tissue. Nature 317.

Shimatsu A., Rotwein P. 1987. Masaic evolution of the insulin like growth factors.. Organization, sequence, and expression of the rat IGF-I gene. J. Biol. Chem. 262. 7894.

Shimizu M., Webster G., Morgan D.O., Blau H.M., Roth R.A. 1986. Insulin and insulin-like growth factor receptors and responses in cultures human muscle cells. Am J. Physiol. 251. E611

Soares M.B., Turken A., Ishii D., Mills L., Epikopou V., Cotter S., Zeitlin S., Esfratiadis A. 1986. Rat Insulin Growth Factor II Gene. J. Mol. Biol. 192. 737.

Southern E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98. 503.

Stempien M.M., Fong N.M., Rall L.B., Bell G.I.. 1986. Sequence of a placental cDNA encoding a mouse IGF-II precursor. DNA. 5. 357.

Straus D.S. 1984. Growth stimulatory actions of insulin in vitro and in vivo. Endocrine Reviews. 5.356.

Straus D.S., Williamson R.A., 1978. Actions of insulin-like growth factors. J. Cell Physiol. 97. 189.

Straus D.S.. 1981. Effects of insulin on cellular growth and proliferation. Life Sci. 29. 2131.

Tally M., Li C.H., Hall K. 1987. IGF-2 stimulated growth mediated by the somatomedin Type-II receptor. Biochem Biophys Res Commun. 148. 811

Tavakkol A. Simmen F. A., Simmen R.C.M. 1988. pIGF-I: cDNA cloning and uterine expression of messenger ribonucleic acid encoding evolutionary cinserved IGF-I peptides. Mol Endocrinol 2. 674.

Tricoli J.V., Rall L.B., Karakousis C.P., Herrara L., Petrelli N.J., Bell G.I., Shows T.B. 1986. Enhanced level of insulin like growth factor messenger RNA in human colon carcinomas and liposarcomas. Cancer Res. 46. 6169.

Tricoli J.V., Rall L.B.,Scott J.,Bell G.I., Shows T.B. 1984. Localization of insulin-like growth factor genes to human chromosomes 11 and 12. Nature. 310. 784.

Ueno T., Takahashi K., Matsuguchi T., Endo H., Yamamoto M. 1987. A new leader exon identified in the rat insulin-like growth factor II gene. Biochem. Biophys. Res. Comm. 148 . 344.

Ueno T., Takahashi K., Matsuguchi T., Endo H., Yamamoto M. 1988. Transcriptional deviation of the rat IGF-II gene initiated at three alternative leader exons between neonatal tissues and ascites hepatomas. Biochim. Biophys. Acte. 950. 411.

Ullrich A., Bell J.R., Chen E.Y., Harrera R., Petruzzelli L.M., Dull T.J., Gray T.J.,Coussens L., Liao Y.C., Tsubokawa M., Mason A., Seeburg P.H., Grunfeld C., Rosen O.M., Ramachandran J. 1985. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature. 313. 756

Ullrich A., Gray A., Tam A.W., Yang-Feng T., Tsubokawa M., Collins C., Henzel W., Le Bon T., Kathuria S., Chen E., Jacobs S., Francke U., Ramachandran J.,Fujita Yamaguchi Y. 1986 Insulin-like growth factor-I receptor primary structure: comparison with insulin receptor suggest structural determinants that define structural specificity. EMBO.5. 2503

Valentino K.L., Pham H., Ocrant I., Rosenfeld R.G. 1987. Distribution of insulin like growth factor II receptor immunoreactivity in rat tissue. Proc. Natl. Acad. Sci. USA. 84. 1590.

Venkatasubramanium K., Audhya T., Goldstein G. 1986. Binding of thymopoitin to the acetylcholine receptor. Proc. Natl. Acad. Sci. USA. 83. 3171

von Figura K., Hasilik A. 1986. Lysosomal enzymes and their receptors. Ann. Rev Biochem. 55. 167.

Verspohl E.J., Roth R.A., Vigneri R., Goldfine I.D. 1984. Dual regulation of glycogen metabolism by insulin and insulin-like growth factors in human hepatoma cells (HEP G2). J. Clin. Invest. 74. 1436

Wilson D.M., Thomas J.A., Hamm T.E. Jr, Wyche J., Hintz R.L.,Rosenfeld R.G. 1987. Transplantation of insulin-like-growth-factor-II secreting tumors into nude rodents. Endocrinology. 120. 1896.

Wheeler F.B., Santora A.C., Elsas L.J. 1980. Evidence supporting a two receptor model for insulin binding by cultured embryonic heart cells. Endocrinology. 107. 195.

Whitfield H.J., Bruni C.B., Frunzio R., Terrell J., Nissley P., Rechler M.M. 1984. Isolation of a cDNA clone encoding rat IGF-II precursor. Nature. 312. 277.

Wyche J.H., Noteboom W.D. 1979. Growth regulation of cultured human pituitary cells by steroidal cumpounds in defined medium. Endocrinology 104. 1765.

Zapf J., Froesch E.R., Humbel R.E. 1981. The insulin-like growth factors (IGF) of human serum. Chemical and biological characterisation and aspects of their possible physiological role. Curr Top Cell Reg. 19. 257.

Zick Y., Sasaki N., Rees-Jones R.W., Grunberger G., Nissley S.P., Rechler M.M. 1984. Insulin like growth factor-I stimulates tyrosine kinase activity in purified receptors from a rat liver cell line. Biochem Biophys Res Commun. 119 . 6.

Zumstein P.P., Luthi C., Humbel R.E. 1985. Aminoacid

sequence of a pro form of insulin like growth factor II. Proc. Natl. Acad. Sci. USA. 82. 3169.