THE EFFECT OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I), AND AN IGF-I-LIKE FACTOR SECRETED BY HUMAN LUNG FIBROBLASTS, ON THE GROWTH OF HUMAN LUNG CARCINOMA CELLS IN VITRO

By

David P. Ankrapp

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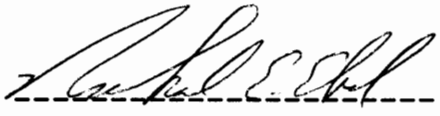
in

BIOCHEMISTRY AND NUTRITION

APPROVED:

David R. Bevan, Chairman



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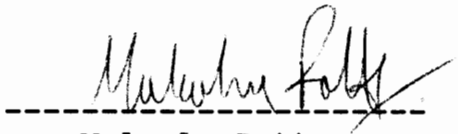

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(ABSTRACT)

The concentration of insulin-like growth factor I (IGF-I) in tissue taken from human non-small cell lung carcinoma (NSCLC) is 1.4- to 7-fold higher than the concentration of IGF-I in the surrounding normal lung tissue and therefore IGF-I may be involved in the growth of NSCLC. In this study it was determined that NSCLC cell lines (A549, A427, SK-LU-1) expressed the type I IGF-I receptor protein and IGF-I stimulated the proliferation of low density plated (2000 cells/cm^2 growth area) carcinoma cells by 1.6- to 3-fold above control after a four day incubation period under serum-free conditions (A549, A427) or in the presence of 0.25% serum (SK-LU-1). In addition, when added to detergent-solubilized type I IGF receptors from A549 cells, IGF-I stimulated [1] a dose-dependent increase in the auto-phosphorylation of the type I IGF receptor, and [2] a dose-dependent increase (1.5- to 4-fold) in the phosphorylation of a tyrosine kinase-specific substrate. These results suggest that the growth promoting activity of IGF-I for the lung carcinoma cells was mediated through the activation of

the type I IGF receptor. Western blot data showed that IGF-I was not secreted by A549 cells, however, immunoreactive IGF-I-like proteins were present in the serum-free medium conditioned by human adult (CCD-19Lu) and fetal (WI-38) lung fibroblasts. The secretion of the immunoreactive IGF-I-like protein by the CCD-19Lu cells was dependent on the passage level of the fibroblasts. Media conditioned by confluent lung fibroblast cultures (LFCM) at population doubling levels between 28 and 30 contained an 8 kD immunoreactive IGF-I-like protein and the majority of the collections of LFCM contained the 8 kD IGF-I-like protein and an immunoreactive IGF-I protein of 13-15 kD. In addition, some collections of LFCM contained the two IGF-I-like proteins described above as well as an immunoreactive IGF-I protein of 17-20 kD. Conditioned media obtained from fibroblast cultures at doubling levels of 32 to 34 contained only the 8 kD IGF-I-like factor, and immunoreactive IGF-I was absent in media from cultures at population doubling levels above 34. The 8 kD IGF-I-like factor promoted the serum-free growth of A549 cells (2-fold increase in cell number over control after 4 days) and stimulated [1] the autophosphorylation of detergent-solubilized type I IGF receptors from A549 cells, and [2] a dose-dependent increase (1.5- to 3.5-fold) in the phosphorylation of a tyrosine kinase-specific substrate by detergent-solubilized type I receptors from each lung

carcinoma cell line. The cell proliferation and type I receptor kinase stimulatory effects of the 8 kD IGF-I-like factor were neutralized by an anti-IGF-I antibody suggesting that the fibroblast-derived factor mediated its activity via the IGF-I receptor. Our data show that IGF-I, and an IGF-I-like factor secreted by human adult lung fibroblasts, stimulate the proliferation of NSCLC-derived cell lines *in vitro* and thus the fibroblast-derived IGF-I could play a role in the growth of NSCLC *in vivo*.

ACKNOWLEDGEMENTS

I would like to thank Dr. David R. Bevan for his patience, guidance, and support throughout the course of my Ph.D. work. His willingness to allow me to pursue a project out of his realm of expertise was the most important factor in my development as a scientist. For that, I will be forever grateful. I would also like to thank the members of my committee, Dr. Richard E. Ebel, Dr. Malcolm Potts, Dr. Thomas O. Sitz, and Dr. Hugo P. Veit, for their helpful comments and suggestions. I thank Dr. Peter J. Kennelly for teaching me the "ropes" concerning the protein kinase assay; his enthusiasm for kinases and phosphatases introduced me to an area of biochemistry which will have an influence on my future endeavors in cancer research. I wish to thank Dr. Pat Bender, Dr. Ed Bunce, and Dr. Walter Niehaus for many insightful discussions about science and non-science issues. I especially thank Dr. Tom Keenan for his moral (and financial!) support during the last year of my stay at Virgina Tech. I'd like to thank Barbara Spence for her wonderful cakes and friendship, and Dr. Deb Ghosal for his ever cheerful disposition. Thanks to all my friends and colleagues in this Department; may peace be with you always. Finally, I would like to thank my dear wife Dawn whose love and encouragement kept me going through the rough times; the completion of this project is due to her strength as well as my own.

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LIST OF ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
BME	Beta-mercaptoethanol
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribonucleic acid
CPM	Counts per minute
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
g	Force of gravity
GH	Growth hormone
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IGF-BP	IGF binding proteins
kb	Kilobases
kD	Kilodaltons
Kd	Dissociation constant
Km	Michaelis constant
LFCM	Lung fibroblast-conditioned medium
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor

PMSF	Phenylmethylsulfonylfluoride
RNAse	Ribonuclease
SDS	Sodium dodecyl sulfate
SFM	Serum-free medium
TCA	Trichloroacetic acid
TGF-alpha	Transforming growth factor-alpha
TGF-beta	Transforming growth factor-beta
Tris	Tris(hydroxymethyl)aminomethane
UT	Untranslated sequence
Vmax	Maximum velocity
Vo	Void volume

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INTRODUCTION

Human bronchogenic tumors are sometimes broadly grouped as either small cell lung carcinomas (SCC) or non-small cell lung carcinomas (NSCLC) based on morphological and biochemical criteria (1). These neoplasms arise from the epithelium of the tracheobronchial tree. Of particular interest is that unlike SCC, NSCLC are generally associated with a prominent fibrotic stroma (2). This feature can be reproduced when cell lines derived from SCC and NSCLC are inoculated into athymic mice (3). All tumors derived from the NSCLC cell lines display an extensive fibrotic stroma which is absent in tumors derived from the SCC cell lines. It has been suggested that the presence of stroma, in part, may explain some of the biologic and clinical differences between NSCLC and SCC (3). The differences in the histology of NSCLC and SCC with respect to the presence of stroma may be related to the differential expression of growth factors by the lung carcinoma cells (4). Cell lines derived from NSCLC express mRNA transcripts for platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-beta) (4,5); both factors are potent mitogens for fibroblasts (5,6). Transcripts for these factors are not expressed by SCC cell lines (4).

Of particular importance to the present study, mRNA

transcripts for IGF-I were not detected in any of the NSCLC cell lines tested (4), although the concentration of IGF-I is 1.4- to 7-fold higher in tissue from NSCLC relative to the concentration of IGF-I in normal lung tissue taken from the same patient (7). The higher concentration of IGF-I in the neoplastic tissue is believed to be indicative of the role of IGF-I in promoting the growth of NSCLC in vivo. The lack of expression of mRNA transcripts by NSCLC cells in vitro could indicate that the source of IGF-I in NSCLC in vivo may come from a cell type other than the neoplastic lung epithelial cells.

IGF-I has been implicated in the growth of a variety of tumors (8). The mode of IGF-I action may be autocrine, i.e. IGF-I stimulates the proliferation of the cells that produce the growth factor, or paracrine, i.e. IGF-I released from one cell type exerts its biological activity on a dissimilar cell (9). In breast cancer, mRNA transcripts for IGF-I and IGF-II are localized to the fibrotic stroma (10) yet type I IGF receptors are present predominantly in the epithelial component of the tumors (11). Therefore, IGF-I may be acting as a paracrine factor to stimulate the growth of the neoplastic mammary epithelial cells (11). Because fetal lung fibroblasts secrete IGF-I (12,13), and a fibrotic stroma is characteristic of NSCLC (2), it is possible that the IGF-I

in NSCLC is derived from the stromal fibroblasts. In this regard, IGF-I secreted by the lung fibroblasts would act in a paracrine manner to stimulate the growth of the neoplastic lung epithelial cells.

The goals of this project were:

[1] To determine whether IGF-I could stimulate the growth of lung carcinoma cells of NSCLC origin.
[2] To determine whether lung fibroblasts secreted IGF-I which in turn could stimulate the growth of the lung carcinoma cells.

The literature review focuses on the biochemistry of IGF-I with respect to its primary structure, gene organization and expression, and interaction with the cellular mediator of the biological actions of IGF-I, the type I IGF receptor.

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LITERATURE REVIEW

A. IGF-I: Mediator of Growth Hormone Effects on Tissue

The IGFs (IGF-I and IGF-II) were discovered as three separate biological activities present in serum and media conditioned by rat liver cells *in vitro*. Sulfation factor was the operational name given by Salmon and Daughaday in 1957 (14) to a factor in rat serum which stimulated the uptake of sulfate into cartilage. Sulfation factor was not present in serum from hypophysectomized rats, but could be restored in the serum of these animals by treatment with growth hormone (GH). Since GH itself had no direct effect on the stimulation of sulfate incorporation into cartilage, the investigators concluded that the growth-promoting effects of growth hormone *in vivo* was mediated via the induction of sulfation factor. At about the same time, Leonards (15) and Froesch *et al*. (16) demonstrated that antibodies to insulin could not completely suppress all of the insulin-like activity in human serum. These activities include the stimulation of glycogen and lipid synthesis, cellular uptake of glucose and amino acids, and stimulation of cell proliferation. The investigators concluded that the factor(s) responsible for the insulin-like activity was distinct from insulin itself and Froesch et al (16) termed the activity in serum as the "non-suppressible insulin-like

activity" (NSILA).

In the early 1970's, Temin and co-workers (17,18) discovered a factor in serum and in medium conditioned by rat liver cells in vitro that stimulated the proliferation of chicken embryo fibroblasts and had insulin-like activity. They termed the growth factor "multiplication stimulating factor" (MSA). It was later determined that the biological properties of MSA could not be distinguished from sulfation factor or NSILA and eventually the generic term "somatomedins" was designated to replace MSA, NSILA, and sulfation factor for the activities in serum that mediated the growth effects of GH on somatic tissue and displayed insulin-like properties (19,20). By 1978, the activities of the somatomedins were determined to result from two factors; the primary structure of each factor was found to be 40-50% identical to insulin (21-23). These factors were renamed insulin-like growth factors I and II, which is now the accepted designation for the somatomedins (24).

The expression of the genes for IGF-I and IGF-II is tissue and developmentally specific (25,26). IGF-II is the predominant somatomedin produced in fetal rat tissue, whereas postnatally, IGF-I is the predominant somatomedin synthesized in tissue and present in the blood (27,28). Likewise in man, mRNA levels of IGF-II exceeds the level of

IGF-I transcripts in all fetal tissues examined (29), but in the adult, IGF-II expression is limited to the liver whereas the IGF-I is expressed in all tissues examined (26). In both man and rat, the rise in postnatal serum IGF-I concentration is concurrent with the increase in the serum level of GH (30,31). IGF-I gene expression in the adult liver, the primary producer of the serum (endocrine) IGFs, is tightly controlled by GH (32,33). GH regulates liver IGF-II gene expression to a lesser extent (33). In the adult human, serum concentrations of IGF-I (normally about 200 ng/ml) reflect GH and nutritional status (34,35). Likewise, serum IGF-I regulates the pituitary expression of GH at the level of transcription as well as secretion (36). Serum levels of IGF-II (normally about 700 ng/ml) are relatively independent of serum GH concentrations (30,31).

Unlike insulin, the IGFs are not found free in the blood and extracellular body fluids. The IGFs are bound to highly specific IGF binding proteins; insulin, despite its structural homology to the IGFs, has no affinity for the IGF binding proteins (37,38). The specific role of the serum IGF binding proteins (IGF-BP), synthesized by the liver, appears to be in the transport of the IGFs to their non-hepatic targets. In addition, serum IGF-BP serve as a biologically inactive reservoir of IGFs; the half-life of IGF-I in serum

increases from 10 min to 10-15 hr when associated with IGF-BP (37). The presence of the IGF binding proteins in extracellular fluids is reflective of the local tissue production of the binding proteins (38). Virtually all cells that secrete and/or respond to the IGFs secrete IGF binding proteins. The IGF binding proteins can either inhibit or potentiate cellular responses to IGF-I dependent on the binding protein. Therefore, the IGF binding proteins play an important, although undefined role in the biology of the IGFs.

GH also regulates IGF-I gene expression in non-hepatic tissue, but it appears that tissue-specific hormones play a more important role in the local regulation of IGF-I biosynthesis (26). For example, estrogens regulate IGF-I gene expression in the uterus, mammary gland, and in bone. In the gonads, IGF-I biosynthesis in granulosa and sertoli cells is stimulated by follicle-stimulating hormone and luteinizing hormone. Parathyroid hormone induces IGF-I expression in chondrocytes and osteoblasts.

The presence of IGF-I mRNA transcripts in all fetal and adult tissues so far examined reflects the endocrine (liver-derived) as well as the locally produced autocrine/paracrine function of the growth factor. The expression of IGF-I mRNA transcripts in fetal human tissue are localized to cells of

mesenchymal origin (39), whereas type I IGF receptors are expressed by both mesenchymal and epithelial cells (40). The detection of immunoreactive IGF-I in media conditioned by fetal human (12) and rat (13) lung fibroblasts, and the immunolocalization of IGF-I in fetal lung epithelium (41) is suggestive of the autocrine and paracrine role of IGF-I in lung development (42).

Emphasis on either the endocrine or local autocrine/ paracrine role of IGF-I as a regulator of growth most likely varies as a function of the physiological state of the organism, although the biological role of IGF-I in vivo is still not completely understood (43). For most cells, IGF-I stimulates insulin-like metabolic effects (such as glycogen metabolism, and glucose and amino acid uptake), but the most important physiological function of IGF-I appears to be the stimulation of cell proliferation. However, it it now clear that IGF-I induces the expression of differentiated functions in many cells. For example, IGF-I stimulates adipocyte differentiation in pre-adipocyte cell lines (44), myotube formation in myoblasts (45), and delta-crystallin gene expression in lens epithelial cells (46). The biological actions elicited by IGF-I are no doubt dependent upon the target cell with respect to the nature of the intracellular signalling pathways that exist in the cell.

B. Structure of IGF-I: Homology to Insulin.

IGF-I is a monomeric protein of 70 amino acids (7646 daltons) with a primary structure that is highly conserved within the animal kingdom (Figure 1). Human, bovine, porcine, and sheep IGF-I are identical, whereas rat and mouse IGF-I have greater than 90% amino acid identity with human IGF-I. Stabilized by three intramolecular disulfide bonds, IGF-I consists of domains B, C, A, and D, in which domains A and B have approximately 50% amino acid identity with the corresponding A and B chains of human insulin (Figure 2). Domain C of IGF-I is structurally similar to the connecting (C) peptide of proinsulin although the domains do not share any amino acid sequence identity. Unique to IGF-I is domain D, an octapeptide carboxyl-terminal extension of domain A.

The alignment of residues 1-29 of human IGF-I (B domain) with the B chain residues 2-30 of human insulin, and alignment of residues 42-62 of IGF-I (A domain) with the A chain residues 1-21 of insulin (Figure 2), reveals that the nine B chain residues which are invariant in all mammalian insulins (B6-8, B11-12, B15, B19, B23-24) are present also in IGF-I (22). Of the ten invariant A chain residues in all insulins, eight are conserved in IGF-I (A1-2, A6-7, A11, A16, A19-20). In addition, the residues in the A and B

B Domain

					5					10					15					20					25
Human	G	P	E	T	L	C	G	A	E	L	V	D	A	L	Q	F	V	C	G	D	R	G	F	Y	F
Bovine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Porcine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-
Mouse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-

B Domain **C Domain**

					29				5						10		
Human	N	K	P	T			G	Y	G	S	S	S	R	R	A	P	Q	T
Bovine	-	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-
Porcine	-	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-
Ovine	-	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-
Mouse	-	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-

A Domain

					5					10					15					20	
Human	G	I	V	D	E	C	C	F	R	S	C	D	L	R	R	L	E	M	Y	C	A
Bovine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Porcine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ovine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mouse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

D Domain

					5			
Human	P	L	K	P	A	K	S	A
Bovine	-	-	-	-	-	-	-	-
Porcine	-	-	-	-	-	-	-	-
Ovine	-	-	-	-	-	-	-	-
Rat	-	T	-	-	-	-	-	-
Mouse	-	T	-	A	-	-	-	-

Figure 1. Amino acid sequence of IGF-I from different mammalian species. Dashes signify residues identical to the top (human) sequence. The peptide domains are indicated above the corresponding sequence. Sequence data from reference 25.

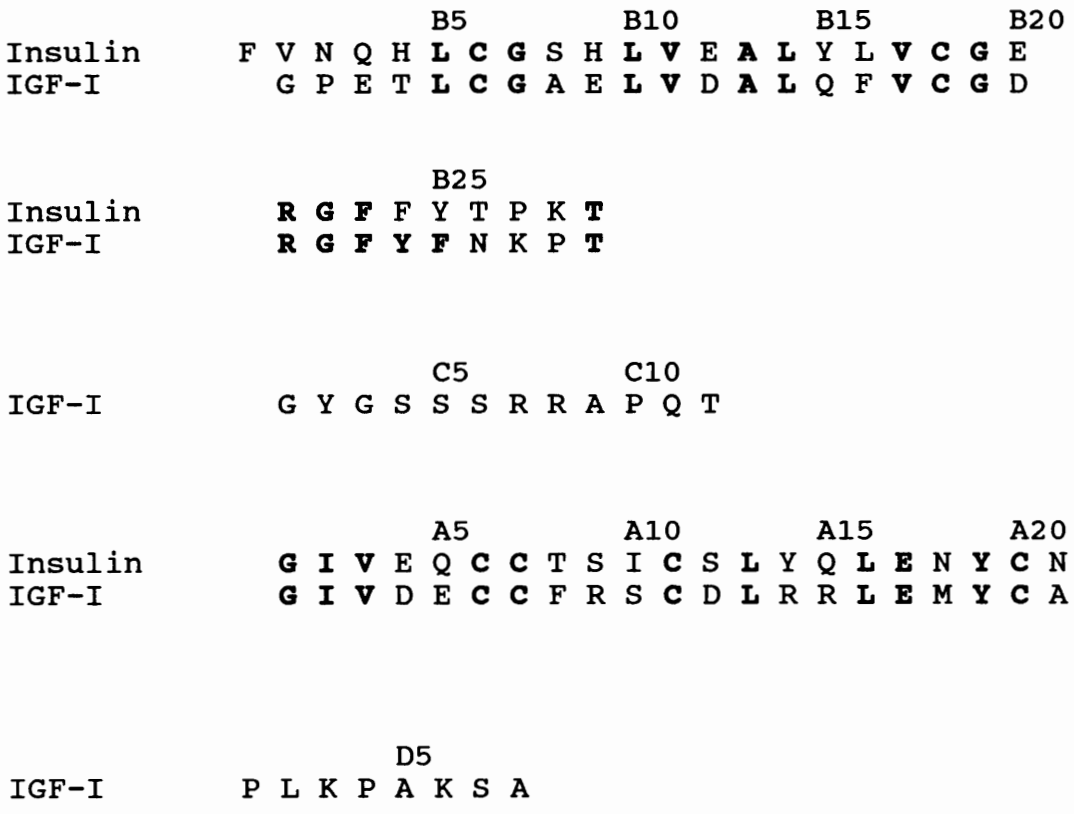

Figure 2. Amino acid sequences of the A and B chains of human insulin and human IGF-I. Numbering of the residues is with respect to the sequence of IGF-I. Bold face residues represent positions of identity between the sequences. Sequence data taken from reference 25.

chains (B10-11, B13-14, B18, A2, A6, A11, and A16) which constitute the hydrophobic interior of human insulin, are conserved in IGF-I. Also, the three glycine residues of the human insulin B chain (B7, B19, and B22) are conserved in the B domain of IGF-I, suggesting that the multiple beta-turns in insulin are structurally conserved in the B domain of IGF-I. Furthermore, the positions of the six cysteine residues of human insulin are invariant in the sequence of IGF-I suggesting that the three intramolecular disulfide bonds in insulin are conserved in IGF-I. Thus, the A and B domains of IGF-I may adopt the same conformation as the corresponding A and B chains of insulin (22). Therefore, even though x-ray crystallographic data for IGF-I is lacking, a three-dimensional model of the tertiary structure of IGF-I has been constructed by computer-assisted molecular modeling (Figure 3) using the structural determinants of porcine insulin as a basis to build the IGF-I model (47,48). The secondary structures of the C and D domains were built using the prediction technique of Chou and Fasman (49) factoring in constraints dictated by the A and B domains. Recently, it was determined by nuclear magnetic resonance that the solution structure of IGF-I is similar to insulin (50). The similiarities in the tertiary structure of IGF-I and insulin may account for the ability of both proteins to

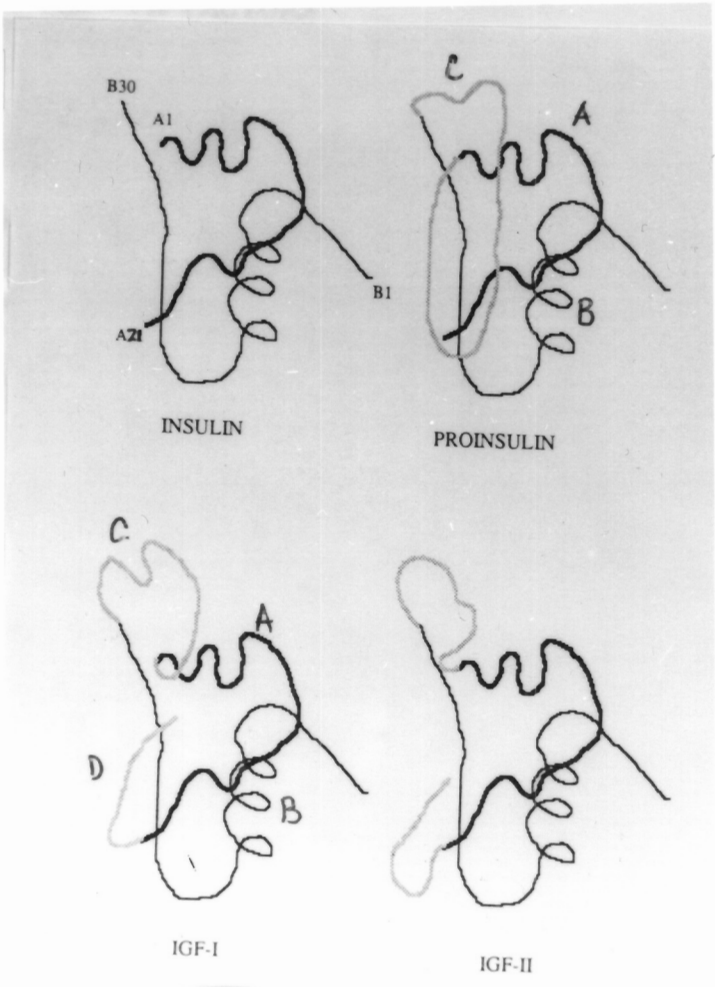

Figure 3. Schematic diagrams demonstrating the structural homology among insulin, proinsulin, IGF-I and IGF-II. The B, A, C and D domains of IGF-I, and the B and A chains and the connecting (C) peptide of proinsulin are indicated. Reproduced from reference 134.

cross-react at the receptor level and elicit similar metabolic and mitogenic responses (48).

Comparisons between the three-dimensional models of insulin and IGF-I together with studies of the biological activities of insulin/IGF-I hybrids and insulin and IGF-I mutants have identified regions within IGF-I important for receptor binding and for the interaction of IGF-I with serum IGF binding proteins (IGF-BP). High affinity binding of IGF-I to serum IGF-BP appears to require amino acid residues localized to the B domain. Insulin does not bind to IGF-BP, however, the substitution of the B chain of insulin for the B domain of IGF-I results in a hybrid (A chain insulin/B domain IGF-I) insulin with an affinity for adult rat and human IGF-BP only 25-fold lower than that of IGF-I (51). Likewise, substitution of the amino-terminal 16 amino acids of the B domain of IGF-I with the corresponding 17 amino acids of the B chain of insulin results in a hybrid (A, C, D domains IGF-I/B chain insulin) IGF-I with no apparent affinity for rat serum IGF-BP (52). Four regions within the amino-terminal 16 amino acid IGF-I B domain differs from the corresponding 17 residues of the B chain of insulin: residues 1-4, 8-9, 12 and 15-16 (Figure 2). The difference at position 12 (IGF-I^{ASP} to $insulin^{Glu}$) is conservative and thus deemed not important for the binding of IGF-I to IGF-

BP. Relative to IGF-I, substitution of the IGF-I B domain residues Glu^3 and Thr^4, with the insulin B chain residue Gln (B3) and Ala, results in a 4-fold decrease in the affinity of the mutant (Gln^3, Ala^4) IGF-I for the IGF-BP (52). Likewise, replacement of the IGF-I B domain residues Gln^{15} and Phe^{16} with the corresponding insulin B chain residues Tyr^{15} and Leu^{16} results also in a 4-fold decrease in the binding of the mutant IGF-I to IGF-BP. However, a mutant IGF-I consisting of all four amino acid substitutions has a 600-fold decrease in its affinity for the IGF-BP. X-ray crystal structure data for porcine insulin indicates that the B chain residues (-)1-5 (Figure 2) have an extended structure and that residues 7-18 form an alpha-helix. The three-dimensional model of IGF-I predicts that the B domain is structurally identical to the insulin B chain and that the combined four residue substitutions would have no effect on the conformation of the B domain except that Phe^{16} faces into the hydrophobic core of IGF-I, whereas Tyr^{15} of insulin and of the (Gln^3, Ala^4, Tyr^{15}, Leu^{16}) IGF-I mutant faces outward into the hydrophilic environment (52). Since the positional shift and change in the orientation of the aromatic ring, by itself, was not sufficient to completely disrupt the affinity of the mutant (Tyr^{15}, Leu^{16}) IGF-I for the serum IGF-BP, it appears that IGF-BP interacts with both

the B domain amino-terminal extended peptide region and Gln^{15} and Phe^{16} near the base of the B domain alpha-helix (52).

The importance of the B domain for binding of IGF-I to serum IGF-BP was further confirmed when a truncated B domain form of IGF-I, which has no apparent affinity for serum IGF-BP, was isolated from fetal (53) and adult (54) human brain and bovine colostrum (55). This IGF-I variant lacks the three amino-terminal residues (Gly-Pro-Glu) of the B domain. With respect to intact IGF-I, the IGF-I varient has a 100-fold decrease in binding affinity for IGF-BP, with no difference in affinity for the type I IGF receptor (56). As will be discussed below (section C), the amino-terminal truncated form of IGF-I may represent the locally active autocrine/paracrine form of the growth factor, whereas the intact protein, which has high affinity for serum IGF-BP, may represent the circulating endocrine form of IGF-I.

Mutations within the B domain of IGF-I as described above, do not effect the binding affinity of IGF-I to the type I IGF receptor (52). This implies that the determinants for IGF-I binding to IGF-BP are different from those required for the interaction with the receptor. Binding of insulin to the insulin receptor requires determinants located on both the A and B chains (57). These include

tyrosine residues B15, B25 (as part of the Phe^{B23}-Phe^{B24}-Tyr^{B25} sequence), and A19 (Figure 2). Because of the predicted structural homology of IGF-I with insulin, the determinants required for binding of IGF-I to the type I IGF receptor may be similar to the putative receptor binding regions of insulin. Surface iodination of free (unbound) IGF-I results in the chemical modification of all three tyrosine residues (B24, C2, and A19; Figure 2) of the protein (58). These data are in agreement with the model of the predicted tertiary structure of IGF-I which shows that the side chain of each tyrosine residue is located on the surface of the protein (48). When IGF-I is bound to the type I IGF receptor, all three tyrosines of the growth factor are protected from the iodination suggesting that these residues are involved in receptor binding (58). The role of the tyrosine residues in the high affinity binding of IGF-I to the type I IGF receptor was confirmed by site-directed mutagenesis studies in which the tyrosines were replaced with non-aromatic residues (59). Relative to IGF-I, single point substitutions (Tyr for Ile or Ser) resulted in a 6-fold (Tyr^{C2}), 18-fold (Tyr^{B24}), and 20-fold (Tyr^{A19}) decrease in the affinity of the mutant IGF-I for the type I receptor. Double mutations involving Tyr^{A19} and either Tyr^{B24} or Tyr^{C2} resulted in a 1200- and 500-fold decrease, respectively, in affinity for the

receptor. The binding affinity of the (Tyr^{B24}, Tyr^{C2}) mutant declined by only 250-fold, indicating the relative importance of Tyr^{A19} in maintaining the high affinity binding of IGF-I to the type I receptor. The high affinity binding of insulin to the insulin receptor is dependent also on Tyr^{A19}, as well as Phe^{B24}, analogous to Tyr^{B24} of IGF-I (57), indicative of the overlapping residue determinants required for binding of the growth factors to their homologous receptors. The IGF-I mutant in which all three tyrosines were replaced had no apparent affinity for the type I IGF receptor.

It could not be determined whether the decrease in the receptor binding affinity of the single, double and triple IGF-I mutants were due to the loss of specific interactions of the tyrosines with the receptor or due to localized main chain perturbations in the receptor binding regions (59). The model of IGF-I predicts that the receptor binding region of the A domain is stabilized by van der Waals forces between Tyr^{A19} and Ile^{A2}; the same residues are responsible for stabilizing the conformation of the insulin A chain (48), and both amino acids are required for the high affinity binding of insulin to the insulin receptor (57). Substitution of Tyr^{A19} with either leucine or serine may have destabilized the conformation of the A domain resulting in

the decreased binding affinity of the IGF-I mutant for the type I receptor.

Substitution of the IGF-I sequence Phe-Tyr-Phe (B23-B25) for the analogous Phe-Phe-Tyr (B23-B25) of insulin does not significantly (< 2-fold decrease) affect the affinity of the IGF-I mutant for the type I receptor (60). This suggests that the Tyr^{B24} hydroxyl group does not directly interact with the receptor and that the decrease in receptor affinity of the Tyr^{B24} mutant (Leu or Ser substitution) is due to the absence of the aromaticity at position 24 required for stabilization of the main chain conformation of the B and C domain receptor binding pocket (59).

The 6-fold decrease in receptor affinity of the Tyr^{C2} mutant (59) accounts for some of the loss in the type I receptor affinity (30-fold) of an IGF-I mutant where the C domain was replaced with a four residue glycyl bridge (61). The junction between the B and C domains is predicted to consist of two beta-turns involving residues B27-C3 which brings the side chain of Tyr^{C2} into close position with Tyr^{B24} and presumably stabilizes the type I receptor binding region of the B and C domains (59). The difference in the receptor affinity of the Tyr^{C2} and C domain mutants indicates the importance of the non-tyrosine residues in the C domain for binding of IGF-I to the type I receptor. The affinity of the

C domain mutant for the insulin receptor, serum IGF-BP, and the type II IGF/mannose-6-phosphate receptor does not change, indicating that the glycyl bridge substitution still allows for the proper formation of the IGF-I tertiary structure involving the intact A and B domains (61). Nuclear magnetic resonance determination of the solution structure of IGF-I shows that the C domain lacks any regular secondary structure and thus appears to have little effect on the conformations obtained by the A and B domains (50). Therefore, the decrease in the affinity of the IGF-I mutants for the receptor, due to the tyrosine substitutions in the B and C domains, may have been the result of localized changes in the main chain conformation of the receptor binding regions.

The role of the D domain in the biological activity of IGF-I is not clear. The predicted structure of the D domain indicates that the octapeptide forms an anti-parallel beta-sheet which folds near the type I receptor binding region Phe^{B23}-Tyr^{B24}-Phe^{B25} and thus may influence the binding of IGF-I to the receptor (48). However, relative to IGF-I, deletion of the D region has no significant (< 2-fold) effect on the affinity of the IGF-I mutant for the type I receptor or for serum IGF-BP, although the affinity of the IGF-I mutant for the insulin receptor increases 2-fold, suggesting that the

function of the D region is to sterically hinder the binding of IGF-I with the insulin receptor (61).

C. The IGF-I Gene: Developmental and Tissue-Specific Expression and Alternative Splicing of the Primary RNA Transcript.

The human IGF-I gene has been mapped to chromosome 12 and spans more than 90 kb (62). The gene consists of 6 exons separated by intervening sequences of 1.5 kb to greater than 65 kb (Figure 4). The exact size of the gene is unknown due to the uncertainty in the length of the intron between exons 3 and 4. Expression of the IGF-I gene results in a primary transcript that undergoes alternative splicing to generate mRNA species encoding distinct prepro-IGF-I proteins designated as IGF-IEa and IGF-IEb (63). The transcript encoding for IGF-IEa consists of exons 1 or 2, 3, 4, and 6, whereas the transcript for IGF-IEb consists of exons 1 or 2, 3, 4, and 5 (Figure 4). The mature 70 amino acid protein is generated following proteolytic processing at both the amino- and carboxyl-terminus.

Exons 1 and 2 encode alternative 5' untranslated sequences (UT) as well as the sequence for the first 21 amino acids (Exon 1) or 5 amino acids (Exon 2) of the signal peptide. Solution hybridization studies have shown that

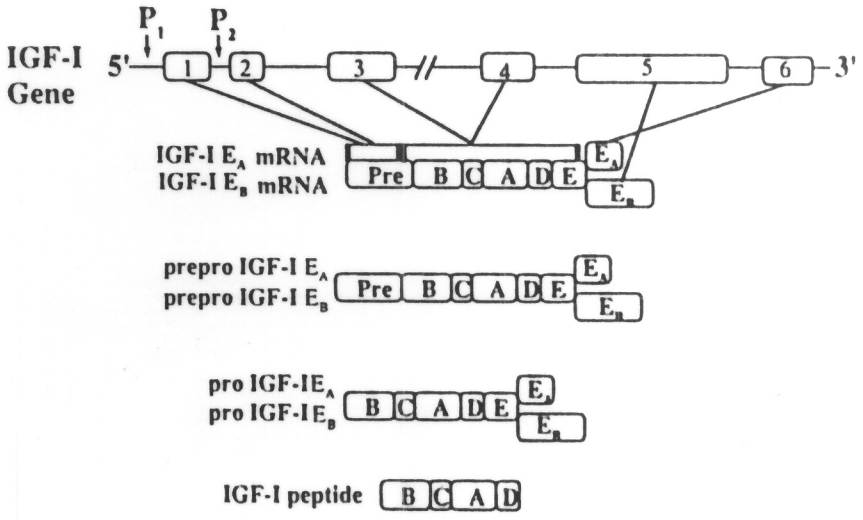

Figure 4. Schematic diagrams of the human IGF-I gene, the alternatively spliced mRNA transcripts for IGF-IEa and Eb, prepro- and pro- translation products containing the signal peptide (prepro-) and E domains (prepro-and pro-), and final processed form of mature IGF-I. Reproduced from reference 135.

approximately 3% of human liver IGF-I mRNAs contain the exon 2 encoded alternative 5' UT and amino-terminal leader peptide sequence (64). Sequence analysis of exon 2 shows substantial nucleotide homology (> 80%) to the alternative leader sequence (exon 2) of the rat IGF-I gene, whereas exon 1 has substantial homology to the primary leader exon (exon 1) of the rat gene (discussed below). Each leader exon contains multiple transcription start sites upstream from the predicted AUG translation initiation codon (64,65). Nucleotide sequence analysis of a potential promoter region 5' to each leader exon indicates that the putative IGF-I promoters belong to the class of TATA-less, non-GC rich promoters (65).

Exon 3 encodes for the carboxyl-terminal 27 amino acids of the signal peptide and the first 25 amino acids of the mature peptide B domain. The remaining amino acids of domain B, and the amino acids of the mature peptide domains C, A, and D are encoded in Exon 4. In addition, Exon 4 encodes for the first 16 amino acids of the carboxyl-terminal E domain. Therefore, the first 134 (exon 1 transcripts) or 118 (exon 2 transcripts) amino acids of the prepro-proteins are identical. The difference between the prepro-IGFs occurs at the carboxyl-terminal extension of domain E.

Exons 5 and 6 each encode distinct E domain extension peptides, 3' UT sequences, and polyadenylation addition signals and sites. Exon 5 encodes for the carboxyl-terminal extension of domain E of IGF-IEb (61 amino acids), whereas Exon 6 encodes for the E domain extension of IGF-IEa (19 amino acids). There is no amino acid sequence identity between the E domain extension peptides as predicted by analysis of the cDNAs. In addition, the 3' UT sequence of exon 5 contains only one polyadenylation signal sequence and downstream (16 bp) polyadenylation addition site, whereas the 3' UT sequence of exon 6 encodes two polyadenylation signal sequences, separated by about 6 kb, and their respective downstream (13 to 19 bp) polyadenylation addition sites. In the liver, the multiple mRNA transcripts (7.0-7.6 and 0.8-1.2 kb) for IGF-IEa are due to the use of the alternative polyadenylation signal sites (64). Poly-adenylation at the upstream site gives rise to the 0.8-1.2 kb transcript, whereas polyadenylation at the downstream signal site results in the 7.0-7.6 kb transcript. In non-hepatic tissue, the primary transcript for IGF-IEa is the 7.0-7.6 kb mRNA. The biological significance of the tissue-specific expression of the multiple IGF-IEa transcripts is not known, although sequence analysis has revealed that the longer transcript is rich in the sequence $A(U)_nA$ which

appears to have a destabilizing effect on eukaryotic mRNAs especially transcripts encoding growth-related proteins (66). The primary transcript encoding IGF-IEb in hepatic and non-hepatic tissue is 1.3-1.5 kb.

In vitro transcription/translation studies have indicated that the primary translation products of human liver exon 1 transcripts encoding for IGF-IEa and IGF-IEb contain 153 (17.5 kD) and 195 (22.5 kD) amino acids, respectively (63). This indicates that translation is initiated at the first in-frame AUG codon in exon 1. Each prepro-protein undergoes cotranslational processing in the endoplasmic reticulum that results in the cleavage of the amino-terminal 48 amino acid signal peptide and produces the pro-IGF-I peptides of 105 amino acids (IGF-IEa, 12.5 kD) and 148 amino acids (IGF-1Eb, 17.5 kD). The proteolytic removal of the E domain for both prohormones is post-translational, however it is not clear if the processing occurs intracellularly, or if the proteolysis is a post-secretory event. High molecular weight IGF-I proteins (16-26 kD) have been found in the media of human cells in vitro (67-69), and IGF-IEa has been detected in human serum of patients with chronic renal failure using an antibody directed against the carboxyl-terminal extension region of the prohormone Ea domain (70).

As was discussed above, exon 2 encodes the first 5 amino acids of the amino-terminal leader peptide resulting in a prepro-IGF-I with a signal peptide of 32 amino acids rather than the 48 amino acid leader peptide encoded by exon 1 transcripts. The biological significance of the alternatively used 5' leader exons as well as the expression of the different prohormone forms of IGF-I are not yet known, but studies investigating the regulation of expression of the rat IGF-I gene has resulted in a number of interesting hypotheses as discussed below.

The complex organization of the IGF-I gene is highly conserved among different mammalian species (26). Of these, the rat IGF-I gene has been extensively characterized. The rat gene spans more than 70 kb and consists of 6 exons subdivided by 5 introns of various size (2.0-50 kb) (71,72). Mature IGF-I (domains B, C, D, and A) is encoded by exons 3 and 4. In addition, exon 3 encodes the carboxyl-terminal 27 amino acids of the signal peptide, whereas exon 4 encodes the amino-terminal 16 amino acids of the E domain. The E domain extension peptides are encoded by exon 5 (IGF-IEb) and exon 6 (IGF-IEa). Exon 6 encodes also the 3' UT region. Exons 1 and 2 encode for alternative 5' UT regions and either the amino-terminal 21 amino acids (exon 1) or 5 amino acids (exon 2) of the signal peptide.

As with the human gene, expression of the rat IGF-I gene results in a primary transcript that undergoes alternative splicing in a tissue and developmentally-specific manner to generate mRNAs that encode for either IGF-IEa or IGF-IEb, but the genesis of the rat transcripts for the prepro-IGFs result solely from the inclusion/ exclusion of exon 5. IGF-IEa transcripts contain only exon 6, whereas IGF-IEb transcripts contain exons 5 and 6. In contrast, as described above, the human IGF-IEa and Eb transcripts are derived from the mutually exclusive splicing of exons 5 or 6 to exon 4. There is considerable nucleotide sequence homology (60-90%) of the rat gene to the human gene dependent upon which exon/intron is used in the homology comparison (71,72), and as described above, extensive amino acid identity (96%) exsists between the rat and human forms of mature IGF-I.

The predominant mRNA transcripts in all rat tissues encode for IGF-IEa (72). Transcripts for IGF-IEb are found in lower abundance (< 5% of the total IGF-I mRNA) in all tissues examined, but are expressed at much higher levels (10% of total IGF-I mRNA) in the liver. In hypophysectomized rats, the steady-state levels of liver IGF-IEb transcripts increase 7- to 8-fold in response to growth hormone therapy, whereas the steady-state levels of the transcripts for IGF-

IEa are significantly less effected by growth hormone (2-fold increase over controls) (72). Furthermore, growth hormone does not significantly increase (< 2-fold) the abundance of IGF-IEb transcripts in non-hepatic tissues, whereas the abundance of IGF-IEa transcripts increased at least 2-fold in all tissues examined. It is postulated that IGF-IEb may represent the endocrine form of IGF-I, and IGF-IEa (in the liver and extra-hepatic tissues) represents the locally active autocrine/paracrine IGF-I (73). As with the human prepro-IGF-I proteins, cotranslational processing at the amino-terminus, and the post-translational proteolytic removal of the E domain of the prepro-peptides generates the mature form of rat IGF-I.

With respect to the 5' terminus of the rat gene, it was shown by sequence analysis that the alternative leader exons 1 and 2 contain multiple transcription initiation start sites and a TATA-less promoter region located just upstream of the 5'end of each exon (72). Each leader exon contains multiple in-frame AUG translation initiation codons, but <u>in vitro</u> translation studies have indicated that the primary product of exon 1 transcripts contains a 48 amino acid signal peptide whereas proteins translated from exon 2 transcripts contain a 32 amino acid signal sequence. In addition, translation initiation of exon 1 and 2 transcripts

can occur at the first AUG codon within exon 3 which generates a prepro-IGF-I with a signal peptide of 22 amino acids. The carboxyl-terminal 19 amino acids of the signal sequence contains the structural features typical of eukaryotic signal peptides (74). There is greater than 90% amino acid identity in the sequence of the 48 amino acid signal peptide between the rat and human prepro-IGFs, thereby suggestive of the yet unknown biological importance of the unusually long 48 amino acid signal sequence (71).

The use of the alternatively spliced leader exons appears to be developmentally regulated and tissue-specific (75). Exon 2 transcripts are primarily expressed in the liver (approximately 25% of the liver IGF-I mRNA) and expression is tightly regulated by growth hormone (72,75). Transcripts containing exon 1 are the major transcripts found in all tissues (> 90% of total IGF-I mRNA, except in the liver) and steady-state levels are less affected by growth hormone. The developmental regulation of exon 1 and exon 2 containing transcripts in the postnatal liver coincides with the circulating level of growth hormone (26). Exon 1 transcripts increase in abundance before the growth hormone-dependent increase in serum IGF-I concentration is observed. As the serum concentration of growth hormone increases, so does the abundance of exon 2 transcripts and

subsequently, the serum concentration of IGF-I. It is believed that exon 2 transcripts encode for the "endocrine" form of IGF-I (possibly IGF-IEb, see above) whereas exon 1 transcripts encode for an IGF-I (possibly IGF-Ia, see above) which is tissue-specific in its regulation and acts locally in an autocrine/paracrine manner (73).

A mechanism has been proposed by LeRoith and Roberts (73) to explain how the leader exon transcript variants encode for either the endocrine form of IGF-I or the locally active autocrine/paracrine IGF-I. As described above, exon 1 and exon 2 of both the human and rat gene encode for the first 21 or 5 amino acids of the signal peptide, respectively. The remaining 27 amino acids of the signal peptide are encoded in exon 3; it is this sequence of amino acids that contains the consensus motif for recognition and cleavage by the signal peptidase (74). According to the mechanism proposed by LeRoith and Roberts (73), exon 1 and exon 2 transcripts undergo differential cotranslational processing to generate amino-terminal truncated (exon 1 transcripts) and intact (exon 2 transcripts) prohormones. The differential processing would be dictated by structural features within the amino-terminal 21 amino acid sequence of the 48 residue signal peptide which would alter the site of cleavage by the signal peptidase and result in a pro-IGF-I

lacking the first three amino acids of the B domain. These structural motifs would be lacking in the 32 amino acid signal peptide encoded by exon 2 transcripts and thus the signal peptidase would cleave the prepro-IGF-I at the consensus signal site resulting in the intact pro-IGF-I. Recent studies have shown that structural motifs within the amino-terminus of eukaryotic signal peptides ultimately determine the site of cleavage by the signal peptidase (74).

As was described above, cell-free translation/microsomal processing of exon 1 transcripts from human liver results in the generation of intact prohormone forms of IGF-IEa and IGF-IEb. Therefore, if the proposed mechanism of differential processing is correct, structural motifs within the 48 amino acid signal sequence, as well as undefined host factors, may be needed to generate the truncated form of IGF-I. An alternative mechanism has been proposed which suggests that the amino-terminal truncation of the B domain is a post-translational, intracellular event (26). In this mechanism, IGF-IEa is the substrate for the putative protease since IGF-IEa transcripts are predominant in non-hepatic tissue and thus represent the locally active autocrine/paracrine IGF-I, whereas IGF-IEb is the endocrine IGF-I since its expression is approximately 5-fold higher in the liver than in non-hepatic tissue. Regardless of the

mechanism, as described in section B, the physiological difference between the truncated and intact forms of IGF-I is that amino-terminal truncation of the B domain reduces the affinity (100-fold) of IGF-I to serum IGF-BP without affecting its affinity for the IGF-I receptor (56). Therefore, the secreted truncated IGF-I would remain local whereas the secreted intact IGF-I would immediately interact with IGF-BP and be carried in the circulation. To date, tissue IGF-I, as opposed to circulating IGF-I, has been found to consist solely of the truncated form of mature IGF-I (26).

Alternative polyadenylation signal sites within the 3' UT of exon 6 appear to provide an additional avenue of post-transcriptional regulation of rat IGF-I biosynthesis. IGF-I transcripts in the liver with a given E domain coding sequence and leader exon range in size from 0.8-1.2 kb up to 7.0-7.5 kb. The difference in the size of the transcripts is due to the length of the 3' UT region. Using the RNAse protection assay coupled with sequence analysis, it was determined that the multiple transcripts are derived by the use of at least two polyadenylation signal sites (72,76) although the 3' UT region encodes four potential polyadenylation signal sequences (71). The length of the 3' UT region appears to dictate the stability of the

transcripts. A single injection of growth hormone to hypophysectomized rats induces a parallel time-dependent increase in the expression of both the 0.8-1.2 kb and 7.0-7.5 kb liver transcripts, yet the half-life of the larger transcript is 4 h versus 14 h for the smaller transcript (77). The larger transcripts are rich in the sequence $A(U)_nA$ which is also present in transcripts from transiently expressed genes such as c-fos and c-myc, although there appears to be no uniquely conserved sequence motif to act as a degradation signal (66). In cell-free translation studies, transcript half-life varies inversely with the frequency of $A(U)_nA$ sequences within the 3' UT region (78). Of interest is that the 7.0-7.5 kb transcripts are the most abundant transcripts in non-hepatic tissue, whereas the 0.8-1.2 kb transcripts are the most abundant mRNAs in liver (76). Physiologically, the predominance of the larger transcripts in extra-hepatic tissues suggest that post-transcriptional control via mRNA stability may be the most important mechanism in the regulation of IGF-I biosynthesis in these tissues (76).

D. Type I IGF Receptor: Structural and Functional Homology to the Insulin Receptor.

The biological actions of IGF-I are initiated following its binding to the type I IGF receptor, a plasma membrane-associated tyrosine-specific kinase which is structurally and functionally similar to the insulin receptor. The type I receptor is a heterotetrameric glycoprotein (Figure 5) comprised of two extracellular alpha subunits (130-140 kD) and two transmembrane beta subunits (85-95 kD). The tetramer is stabilized by interchain disulfide bonds. The alpha subunits of the holoreceptor form a single high affinity ligand binding site (K_d = 0.5-1.5 x 10^{-9} M), although the alpha subunit of an alpha-beta heterodimer can bind IGF-I with 6-fold lower affinity relative to the holoreceptor (79). Each beta subunit consists of an extracellular domain, a single transmembrane domain, and an intracellular tyrosine kinase domain.

The mammalian gene for the type I IGF receptor (mapped to chromosome 15) is greater than 100 kb, consists of 21 exons, and encodes both the alpha (exons 1-11) and beta (exons 12-21) subunits (80). The size and structural organization of the type I IGF receptor gene and the human insulin receptor gene are highly homologous (80,81), underlying the close evolutionary relationship of the two

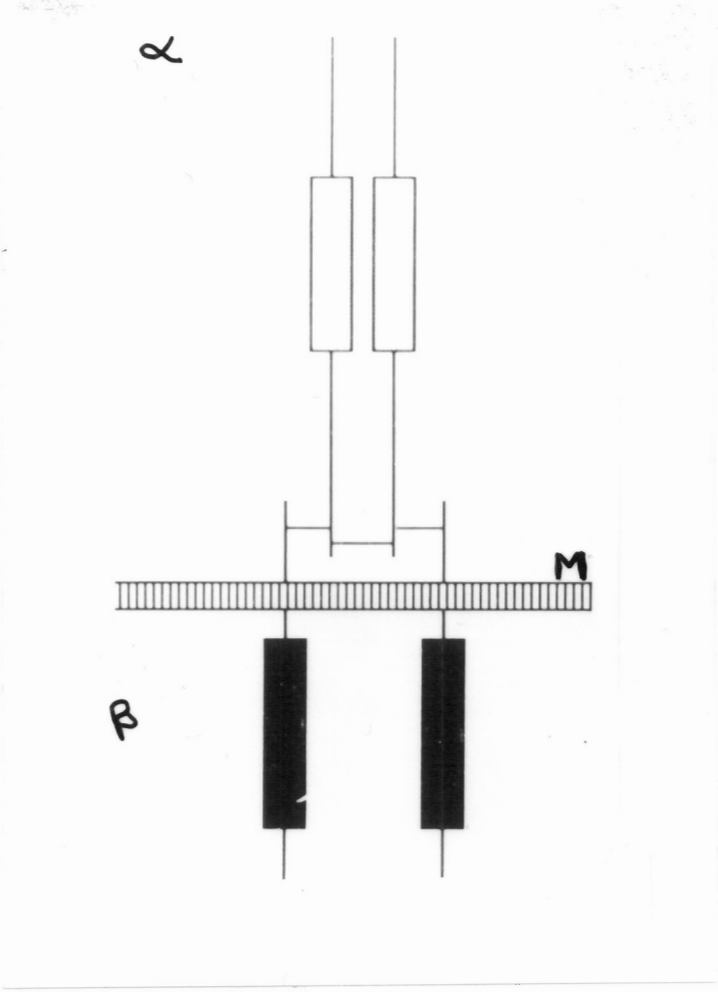

Figure 5. Schematic diagram of the type I IGF receptor. The alpha and beta subunits are indicated. Clear rectangles represent the cysteine-rich domains which form the high affinity IGF-I binding site. Shaded rectangles represent the tyrosine kinase domains. M: membrane. Reproduced from reference 136.

receptors. Twelve out of the 21 exons of the type I receptor gene are identical in size with the homologous exons of the insulin receptor and the sizes of each of the remaining exons (except exons 1 and 21 which encode for the 5' and 3' UT sequence) do not differ by more than 15 nucleotides with the corresponding exons of the insulin receptor (80).

Analysis of the full length cDNA encoding for the type I receptor predicts a prepro-protein of 1367 amino acids (81). The prepro-type I receptor has an overall 50% amino acid identity with the prepro-insulin receptor. The region of highest identity (80-95%) between the receptors comprises the tyrosine kinase domain. The structural organization of the prepro-type I receptor is homologous to the prepro-insulin receptor: NH_2-signal peptide-alpha subunit-beta subunit-COOH. Cotranslational processing within the ER removes the 30 amino acid signal peptide. Analogous to the pro-insulin receptor, the highly basic tetrapeptide sequence Arg-Lys-Arg-Arg is located at the junction of the alpha and beta subunits of the pro-type I IGF receptor. This sequence is the site of proteolytic cleavage which generates the individual subunits of the insulin receptor.

The predicted primary structure of the alpha subunit contains a cysteine-rich (24 cysteinyl residues) domain, which dictates the binding specificity of the type I

receptor for IGF-I (82), and 11 potential N-glycosylation sites (81). The alpha subunit of the holoreceptor is highly glycosylated (as is the extracellular domain of the beta subunit); the molecular weight of the alpha subunit based on cDNA analysis is about 80.5 kD, whereas the alpha subunit of the holoreceptor has a molecular weight of 130-135 kD. More than 90% of the putative N-glycosylation sites are conserved between the type I IGF and insulin receptors. The cysteine-rich domain forms the binding pocket for IGF-I (82). The positions of the cysteinyl residues that make up the cysteine-rich IGF-I binding domain are conserved also in the insulin receptor; this may explain, in part, the cross-reactivity of IGF-I with the insulin receptor. However, the cysteine-rich domain of the insulin receptor (with approximately 50% amino acid identity with the homologous domain of the type I receptor) appears to be of minor importance for the high affinity binding of insulin to its receptor (82). Therefore, despite the structural similarity and high (50-70%) amino acid identity between the insulin and type I IGF receptor alpha subunits, the specificity of the receptors for their respective ligands is determined by separate domains within the alpha subunits. IGF-I and insulin can cross-react with each others receptor at 20- to 100-fold lower binding affinity than for their own receptor.

The transmembrane domain of each beta subunit of the type I holoreceptor is a 24 amino acid alpha-helix; it is not clear the role this region plays in the activation of the intracellular tyrosine kinase following the association of IGF-I to the extracellular binding domain. The structurally homologous transmembrane domain of each beta subunit of the insulin receptor has only 27% amino acid identity with the transmembrane domain of the type I receptor but an equivalent number of residues and the same degree of hydophobicity as determined by hydropathy analysis (81). A recent study into the function of the insulin transmembrane domain with respect to signal transduction has shown that a single point mutation (Val^{938} to Asp) within the transmembrane domain results in the constitutive activation of the receptor tyrosine kinase activity (83). The position of the Val^{1938} in the insulin transmembrane domain corresponds to the position of a valine residue in the transmembrane domain of the c-erbB2/neu tyrosine kinase receptor which is the site for a single base mutation in the nucleic acid sequence of the neu gene that results in a constituitively activated receptor kinase (84). In cells stably transfected with either the wild type (WT) or mutant insulin receptor, the metabolic (glucose transport) and mitogenic (DNA synthesis) responses of cells expressing the

mutant receptor were as high in the absence of insulin as the responses of cells expressing the WT receptor under conditions of maximum insulin stimulation (83). Therefore, the transmembrane domain appears to play an essential role in the intracellular transduction of the insulin signal. The Val^{938} residue occurs in a sequence (Phe-Val-Phe) of similiar hydropathy relative to a corresponding sequence (Val-Leu-Leu, residues 914-916) in the transmembrane domain of the type I receptor (81). It is possible that the transmembrane domains of the insulin and type I IGF receptors share not only structural similarity, but functional similarity as well.

Transduction of the extracellular signal results in the activation of the intracellular tyrosine kinase of each beta subunit. A recent study using kinase-deficient type I receptors demonstrated that most, if not all, of the metabolic and mitogenic actions of IGF-I require an active receptor tyrosine kinase (85). Following ligand binding, the initial event in the signaling process is the auto-phosphorylation of tyrosine residues located within the tyrosine kinase domain of each beta subunit. Auto-phosphorylation activates the phosphotransferase of each beta subunit to catalyze the phosphorylation of tyrosyl residues in various intracellular proteins. With respect to

the insulin receptor, autophosphorylation is predominantly an intramolecular event via trans-phosphorylation (86,87).

Studies with the insulin receptor indicate that tyrosyl residues at positions 1146, 1150, and 1151 (residue numbering as indicated in reference 81) are the major sites of the autophosphorylation reaction (88). All three residues must be phosphorylated in order for the kinase to mediate completely the biological actions of insulin. However, each individual biological response elicited by insulin is determined by the pattern of phosphorylation of the tyrosine residues (89). Phosphorylation of Tyr^{1146} is required for the stimulation of DNA synthesis, whereas phosphorylation of the residues at positions 1150 and 1151 is required for the stimulation of glycogen synthesis. Therefore, multiple intracellular signal transduction pathways with distinct intracellular substrates appear to be required for the mediation of the biological activties of insulin. For the type I receptor, the homologous autophosphorylation sites correspond to tyrosyl residues 1131, 1135, and 1136 (81). Since IGF-I stimulates metabolic as well as mitogenic cellular responses (90), it is conceivable that these activities of IGF-I are mediated by the type I receptor via distinct intracellular signalling pathways. For each receptor, the tyrosine autophosphorylation sites are located

downstream from the consensus sequence (Gly-X-Gly-X-X-Gly followed by 14-20 residues then Ala-X-Lys, where X is any amino acid) for the putative ATP binding site.

The mechanism by which extracellular binding of IGF-I to the type I receptor translates into activation of the intracellular receptor kinase is not clear. For the insulin receptor, the current hypothesis is that insulin binding to the extracellular domain releases the intracellular kinase domains from conformations unfavorable for kinase activity (91). Therefore, the beta subunit is a constitutively activated tyrosine kinase and the alpha subunit negatively regulates the activity of the beta subunit. This implies that the extracellular signal allowing for the release of the inhibitory conformation of the intracellular kinase domains must be transduced via the transmembrane region. As described above, a point mutation within the transmembrane domain of the insulin receptor constitutively activates the receptor tyrosine kinase (83). In addition, limited proteolysis of the insulin receptor removes the bulk of the alpha subunit and results in the ligand-independent activation of the beta subunit tyrosine kinase (92,93). The kinetic properties of the intact insulin-stimulated receptor kinase and the proteolytically-activated receptor kinase are identical with respect to Vmax and Km for a peptide

substrate (92). In addition, sites of autophosphorylation of the tyrosyl residues within the beta subunits are similar for receptors activated by either insulin or proteolysis.

Analogous to the insulin receptor, release of the intracellular kinase domains from inhibitory conformations may account for the activation of the phosphotransferase activity of the type I IGF receptor in response to ligand binding. Chimeric holoreceptors consisting of the alpha subunit of the insulin receptor and the beta subunit of the type I receptor undergo autophosphorylation in response to insulin (94). Furthermore, insulin stimulates a dose-dependent increase in glucose uptake and DNA synthesis in cells expressing the chimeric receptor. Thus, the mechanism by which insulin binding to the extracellular subunits stimulates the intracellular kinase may be similar for the chimeric and insulin receptors. This implies that the beta subunits of the type I receptor are constitutively active kinases negatively regulated by the covalently attached alpha subunits.

The cellular responses elicited by the activated type I receptor kinase are still not completely understood. The activation of the type I receptor kinase results in the phosphorylation, either directly or indirectly, of many intracellular proteins including serine/threonine kinases

(95). It has been suggested that these activated kinases act within several different phosphorylation/de-phosphorylation cascades to transduce the signal from the receptor level to the ultimate effect on cell metabolism.

Specific Aims of the Research Project

The concentration of IGF-I in neoplastic tissue resected from NSCLC is 1.4- to 7-fold higher than the concentration of IGF-I in normal lung tissue (7). In addition, high affinity binding sites for IGF-I, characteristic of the type I IGF receptor, are detected in NSCLC (96). These data suggest that IGF-I may play an important role in the growth of NSCLC. The lack of mRNA transcripts for IGF-I in carcinoma cell lines derived from NSCLC (4) suggests that the source of IGF-I in NSCLC *in vivo* is from a cell type other than the neoplastic epithelial cells. I propose that IGF-I is a potent stimulator of the growth of NSCLC and that the stromal fibroblasts of NSCLC are one source of the IGF-I in these tumors. The experiments described in this study were designed to answer the following specific questions:

[1] Are type I IGF receptors expressed by NSCLC cell lines?
[2] If the type I receptors are expressed by the lung carcinoma cells, does IGF-I stimulate the autophosphorylation of the receptors?
[3] Are the autophophorylated type I receptors activated to phosphorylate a tyrosine kinase-specific substrate?
[4] Can IGF-I stimulate the proliferation of the lung

carcinoma cells?

[5] Is IGF-I secreted by human lung fibroblasts?

[6] Can the fibroblast-derived IGF-I stimulate the auto-phosphorylation of type I IGF receptors from the lung carcinoma cells?

[7] Can the fibroblast-derived IGF-I stimulate the type I IGF receptor from the lung carcinoma cells to phosphorylate the tyrosine-kinase specific substrate?

[8] Can the fibroblast-derived IGF-I stimulate the proliferation of the lung carcinoma cells?

Because of the complexity of the interactions that occur between a tumor and its environment in vivo, it is not completely correct to extrapolate the results of experiments performed with tumor cells in an isolated system in vitro to the situation of the tumor in vivo. However, the data collected in this study may serve to encourage the design of experiments to test whether fibroblast-derived IGF-I stimulates the growth of the neoplastic epithelial cells in vivo, and to further the knowledge of the biology of NSCLC.

EXPERIMENTAL PROCEDURES

Materials

The human lung adenocarcinoma cell lines (A549, A427, SK-LU-1) and the human adult (CCD-19LU) and fetal (WI-38) lung fibroblast cell lines were purchased from the American Type Culture Collection (Rockville, MD). Recombinant human IGF-I, rabbit polyclonal IgG against the alpha subunit of the human IGF-I receptor, and rabbit polyclonal anti-phosphotyrosine antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse monoclonal IgG against human IGF-I (BPL-M23) was purchased from BioDesign International (Kennebunkport, ME). Biotinylated goat anti-mouse IgG antibody, streptavidin-horseradish peroxidase conjugate, Western Blot ECL reagents, and Hyperfilm-ECL were purchased from Amersham (Arlington Heights, IL). Non-specific antibodies (mouse and rabbit IgG), goat anti-rabbit IgG antibody conjugated to horseradish peroxidase, insoluble Protein A, PMSF, ATP, and poly(Glu,Tyr)4:1, were purchased from Sigma Chemical Co. (St. Louis, MO). [gamma-^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear (Boston, MA). Agarose-bound wheat germ agglutinin was from Vector Laboratories (Burlingame, CA). Phenol red-free Eagle's MEM, antibiotics, non-enzymatic cell dissociation buffer, cell

culture grade bovine insulin, and Sephadex gel filtration media were purchased from Sigma. Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). Nunclon cell culture flasks and multi-well plates were purchased from Thomas Scientific (Richmond, VA). Ultrafiltration units, Diaflo YM membranes, and Centricon 3 micro-concentration units were from Amicon (Beverly, MA). General laboratory chemicals were purchased from Sigma and from Fisher Scientific (Pittsburgh, PA).

Methods

Cell cultures. All cell lines were grown in phenol-red free Eagle's MEM supplemented with the non-essential amino acids and containing 20 mM L-glutamine, 25 mM sodium bicarbonate, 10 mM HEPES, 10% FBS, penicillin (100 u/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 ug/ml). This was designated as complete medium. For some experiments the medium lacked serum supplementation (serum-free medium, SFM). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. At confluence, the fibroblast stock cultures were subcultured at 1:2 or 1:4 split ratios and the carcinoma cell lines were routinely subcultured at 1:4 to 1:10 split ratios. For the routine cultivation of stock cultures, the cells were detached from the flasks with

a solution of 0.25% trypsin containing 0.025% EDTA. This solution of trypsin-EDTA also was used to release the carcinoma cells from the flasks prior to the cell count analysis.

Preparation of conditioned media. Confluent fibroblast monolayers at population doubling levels between 28 and 36 were rinsed twice with PBS then incubated in SFM (25 ml/T-150 cm^2 flask). After 24 h this medium was discarded and an equal volume of fresh SFM was added to the cultures. Lung fibroblast-conditioned medium (LFCM) was then collected every 48 h for a six day period with the cultures being replenished with fresh SFM. Each collection of LFCM was centrifuged at 1000xg for 10 minutes to remove cell debris prior to storage at -80°C in the presence of 1mM PMSF.

For the collection of conditioned medium from A549 cultures, the cells were grown to mid-confluence in complete medium then switched to SFM. After 24 h, the medium was discarded and fresh SFM added. Conditioned medium was collected after 48 h and processed as described above. During the course of the incubation, the cells reached confluence. Attempts to collect conditioned medium beyond the first 48 h incubation failed; within 24 h of reaching confluence, the cells began to detach from the plates. The

reason for the cell detachment was not investigated.

Cell proliferation assays. For each carcinoma cell line, confluent stock cultures were trypsinized and the cells replated in 25 cm^2 T-flasks at a density of 50,000 cells/ flask in complete medium. After 24 h, the cultures were rinsed twice with PBS then each culture received 2.5 ml of SFM/0.1% BSA (controls) or media supplemented with 5 ng/ml or 25 ng/ml recombinant human IGF-I (3 flasks/treatment). These concentrations of IGF-I were selected based on the recommendation of the manufacturer for the use of IGF-I as a medium supplement. In some experiments with the A549 cells, the IGF-I was replaced with LFCM protein (10 ug/ml or 25 ug/ml) obtained from fibroblast cultures at population doubling levels between 28 to 30 (see below). Media and growth factor were replenished after 48 h, and at 96 h the cells were harvested using trypsin-EDTA and counted with a hemacytometer. For experiments involving the SK-LU-1 cell line, the assay media were supplemented with 0.25% FBS. The presence of the FBS was required for the SK-LU-1 cells to remain attached to the flasks during the course of the assay incubation period.

For the immunoneutralization experiments, A549 cells were plated at 20,000 cells/well in six-well plates in 1 ml

of complete medium. After 24 h the wells were rinsed with PBS and each well received 1 ml of the appropriate assay medium (3 wells/treatment). Control wells received SFM/0.1% BSA. A second set of wells received medium supplemented with the Sephadex G-100 purified fibroblast IGF-I-like factor (25 ul/well; final assay concentration: 2.5% (v/v)). The remaining wells received the supernatant from the preincubation (24 h, 4°C) of the Sephadex G-100 purified IGF-I-like factor with 50 ug/ml of either BPL-M23 or a non-specific mouse IgG. For the immunoprecipitation, the IGF-I-like factor (0.125 ml) was incubated (24 h, 4°C) with antibody in a final volume of 0.2 ml. After an incubation (2 h, 4°C) with insoluble protein A (10 mg/ml), the immunocomplexes were collected by centrifugation (13,000xg, 10 min) and the supernatant diluted to 5 ml with medium. Each well then received 1 ml of supernatant. If the IGF-I-like protein was not immunoprecipitated, the final concentration of the IGF-I-like protein in the diluted supernatant would be 2.5% (v/v). The assay media were replenished after 48 h, and at 96 h cell numbers were determined as described above.

Type I IGF receptor purification. For each carcinoma cell line, confluent monolayers from eight to ten 150 mm dishes

were detached from the plates with a non-enzymatic cell dissociation buffer. The cells were washed twice with ice-cold PBS then resuspended in ice-cold 50 mM HEPES buffer (pH 7.5) containing 250 mM sucrose and 1 mM PMSF (10 ml/gram cell pellet). All subsequent procedures were carried out at 4°C. The cells were lysed by sonication (3 x 15 seconds, 35% maximum output, Fisher Sonic Dismembrator, model 300) and the lysate cleared of unbroken cells and nuclei by centrifugation (1000xg, 10 minutes). The membrane pellet was obtained following ultracentrifugation (105,000xg, 1 h) of the lysate. The pellet was resuspended in 50 mM HEPES buffer (pH 7.5), containing 150 mM NaCl, 1% Triton X-100, and 1 mM PMSF (final protein concentration of 1-2 mg/ml), and incubated for 1 h with agitation. Non-solubilized material was removed by ultracentrifugation (see above). The type I IGF receptor was enriched from the solubilized membrane preparation by lectin chromatography using wheat germ agglutinin (WGA) coupled to agarose (97). The solubilized protein solution (5 ml, 1-2 mg protein/ml) was passed three times (gravity flow) through a 0.5 ml bed volume (packed resin in a 0.5 cm i.d. Bio-Rad Econo-column) of the WGA-agarose that had been prewashed with 25 ml of 50 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, 0.1% Triton X-100, and 0.01% SDS (buffer A) followed by 25 ml of the above

buffer minus the SDS (buffer B). Following the addition of the sample, the resin was washed with 25 ml of buffer B (0.5 ml/min). Bound protein was eluted from the resin by allowing 0.5 ml of elution buffer (buffer B + 0.5 M N-acetyl-glucosamine) to drain completely into the resin, and the column flow was stopped for 30 to 60 minutes. Following the incubation, 1 ml of elution buffer was passed through the resin (gravity flow) and the effluent was collected as a single fraction. The effluent was designated as the "lectin-purified protein" which contained the type I IGF receptor. The protein concentration of the lectin-purified preparations were 0.1 to 0.2 mg/ml. Aliquots of the preparations were stored at -20°C and subjected to only one freeze-thaw cycle prior to use in the kinase assay. There was no significant decrease in the type I IGF receptor kinase activity of the preparations stored for up to one week under the conditions described above.

Type I IGF receptor immunoblot analysis. Equal amounts of lectin-purified protein (25-40 ug) from each cell line were placed in boiling water for 10 min in the presence of 60 mM Tris-HCl, pH 6.8, 2% SDS, 5% beta-mercaptoethanol (BME), and 15% glycerol. The denatured proteins were subjected to SDS-PAGE (8% separating gels) using the Laemmli buffer system

(98). The upper and lower tank buffer was 25 mM Tris-base, 192 mM glycine, and 0.1% SDS, pH 8.4. The slab gel electrophoresis was carried out using a Hoeffer mini-vertical gel electrophoresis unit at room temperature (25 mA/gel). The separated proteins were then electrophoretically transferred (5 h, 250 mA) to nitrocellulose membranes by the method of Towbin et al. (99) using a Bio-Rad Transblot tank with the electrodes in the standard configuration (separated diagonally by 10 cm). The transfer buffer was 25 mM Tris-base, 192 mM glycine, and 20% methanol, pH 8.4. Prior to the transfer, the gels and the nitrocellulose membranes were soaked in the transfer buffer for about 15 min. Following the transfer, the membranes were incubated (24 h, 4°C) with 0.5 ug/ml of either a rabbit polyclonal IgG against the alpha subunit of the human IGF-I receptor or a non-specific rabbit IgG. All subsequent steps were performed at room temperature. The membranes were washed with 20 mM Tris-HCl buffer (pH 7.5) containing 140 mM NaCl, and 0.1% Tween 20 (TBST) for 30 minutes with three changes of buffer, then incubated (30 minutes) with 1:10,000 diluted anti-rabbit IgG conjugated to horseradish peroxidase. The membranes were washed as described above and bound antibody was detected by chemiluminescence using Amersham ECL western blotting reagents and Hyperfilm-ECL as

described by the manufacturer. Stock solutions of antibodies were diluted with TBST containing 0.1% BSA.

Phosphotyrosine immunoblot of the ligand-activated type I IGF receptor kinase. Approximately 25 ug of lectin-purified protein from A549 cells was incubated at 25°C in a total volume of 0.1 ml in 50 mM HEPES buffer, pH 7.5 containing 50 uM ATP, 20 mM $MgCl_2$, 0.1% Triton X-100, and 0.1% BSA in the absence and presence of recombinant human IGF-I or the IGF-I-like protein secreted by the lung fibroblasts. In some experiments, $MgCl_2$ was replaced with 5 mM $MnCl_2$. The reaction was terminated after 15 min by adding 0.1 ml of ice-cold 20% TCA and the samples were placed on ice. After 30 min, the precipitated protein was collected by centrifugation (13,000xg, 15 min, 4°C) and the top of the protein pellet was washed once with 60 mM Tris-HCl buffer, pH 6.8. The pellets were resuspended in 60 mM Tris-HCl buffer containing 2% SDS, 15% glycerol, with and without 5% BME, then placed in boiling water for 10 min. The sample proteins were fractionated by SDS-PAGE (10% separating gels) then electrophoretically transferred to nitrocellulose membranes as described above. Membranes were incubated (12 h, 4°C) with 1 ug/ml of either a rabbit polyclonal IgG anti-phosphotyrosine antibody or a non-specific rabbit IgG. All

subsequent steps were performed at room temperature. The membranes were washed with TBST (3 x 10 min), then incubated for 60 min with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000 dilution). The membranes were washed, and bound antibody was detected by chemiluminescence as described above. Stock solutions of antibodies were diluted with TBST containing 0.1% BSA.

Type I IGF receptor kinase Assay. Phosphorylation of the tyrosine kinase-specific substrate poly(Glu,Tyr)4:1 was carried out under conditions in which substrate phosphorylation was linear as a function of time (0-15 min) and the rate of phosphorylation was directly proportional to the concentration of the lectin-purified protein in the assay mixture (0 to 50 ug/ml), as described in Results (section 3). The assay was similar to the procedure described by Sasaki et al. (100), but with modifications. Unless otherwise noted, the reactions were carried out within 1.5 ml polypropylene micro-centrifuge tubes at 25°C in 50 mM HEPES buffer (pH 7.5), containing 50 uM ATP (1000-3000 cpm/pmole), 20 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg/ml BSA, 2 mg/ml poly(Glu,Tyr)4:1, in the absence or presence of recombinant human IGF-I, or the immunoreactive IGF-I-like protein secreted by the lung fibroblasts. The lectin-

purified protein was preincubated for 15 min in the absence or presence of growth factor in 45 ul of the HEPES buffer containing $MgCl_2$, Triton X-100, BSA, and ATP (kinase buffer). Substrate phosphorylation was initiated by the addition of poly(Glu,Tyr)4:1 to give a final reaction volume of 50 ul. After 5 to 15 min the reaction was terminated by spotting 30-40 ul of the assay mixture onto a Whatman 3MM filter (2x2 cm) then immersing the filter into a solution (5-10 ml/filter) of 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. The filters were washed for at least 1 hour at room temperature with several changes of the acid solution. The washed filters were rinsed with 95% ethanol, air-dried, and placed into plasic mini-vials containing approximately 2.5 ml of Ecolume. Filter-bound ^{32}P was determined by liquid scintillation counting using a Beckman LS 5801 liquid scintillation counter (2 min count time). Radioactivity associated with the filters in the absence of poly(Glu,Tyr)4:1 was subtracted from that in its presence to yield actual substrate phosphorylation. In general, filter-bound ^{32}P in the absence of poly(Glu,Tyr)4:1 was less than 10% of the radioactivity bound to the filters in the presence of substrate.

For the immunoneutralization experiments, the kinase assay was performed as described above except that the

preincubation was performed in the absence or presence of 25 ug/ml of either the mouse monoclonal IgG against human IGF-I or a non-specific mouse IgG. Preliminary experiments had shown that this concentration of the anti-IGF-I antibody completely inhibited the ability of recombinant human IGF-I (100 ng/ml) to stimulate the kinase activity of type I receptors isolated from A549 cells.

IGF-I immunoblot analysis. Conditioned media from cultures of fibroblasts or carcinoma cells were concentrated by ultrafiltration at 4°C under N_2 gas pressure using an Amicon ultrafiltration unit with a 3 kd cutoff filter (Diaflo YM-3 membrane). The retentates were further concentrated by using Centricon 3 micro-concentration units (3 kd cutoff) as described by the manufacturer. Equal amounts of protein (100 ug) from each collection of LFCM were then placed in boiling water for 10 min in the presence of 60 mM Tris-HCl, pH 6.8 buffer containing 2% SDS and 15% glycerol. The proteins were fractionated by SDS-PAGE (15% separating gels) followed by the electrophoretic transfer of the proteins to nitrocellulose membranes as described above. Membranes were incubated (24 h, 4°C) with 0.5 ug/ml of either a mouse monoclonal IgG against human IGF-I, or a non-specific mouse IgG. All subsequent steps were performed at room

temperature. The membranes were washed with TBST for 30 min with three changes of buffer, then incubated (1 h) with 1:1000 diluted biotinylated goat anti-mouse IgG. The membranes were washed as described above then incubated (1 h) with 1:1000 diluted horseradish peroxidase conjugated to streptavidin. The membranes were washed and bound antibody detected by chemiluminescence as described above. Stock solutions of antibodies and the streptavidin-peroxidase conjugate were diluted with TBST containing 0.1% BSA.

Gel filtration chromatography of fibroblast-conditioned medium. Conditioned medium (200 ml) was concentrated by ultrafiltration as described above and the retentate (2 ml; 1-2 mg protein) was applied to a 1.5 x 90 cm column of Sephadex G-100 equilibrated with 10 mM HEPES buffer (pH 7.5) containing 50 mM NaCl. Fractions (1.5 ml) were collected at a flow rate of about 20 ml/h then concentrated to 0.25 ml using Centricon 3 micro-concentration units. Aliquots (20 ul) of selected fractions were assayed for the presence of IGF-I-like protein by (1) immunoblot analysis and (2) the ability to stimulate the tyrosine kinase activity of type I IGF receptors isolated from A549 cells. Fractions containing immunoreactive IGF-I material were pooled, concentrated

using Centricon 3 micro-concentration units, and stored at -20°C. The column was calibrated using bovine serum albumin (65 kD), turkey egg albumin (45 kD), horse heart cytochrome c (12.5 kD), and bovine insulin (6 kD).

For gel filtration chromatography under acidic conditions, the immunoreactive IGF-I-like protein was first subjected to acid/ethanol extraction to dissociate IGF-I from IGF binding proteins (101). Two ml of acid/ethanol solution (87.5% ethanol/12.5% 2 N HCL) was mixed with 0.5 ml of the pooled Sephadex G-100 chromatography fractions containing the immunoreactive IGF-I-like protein. After 1 h at room temperature, the solution was clarified by centrifugation (10,000xg, 15 min, 4°C) and the supernatant was placed under N_2 to evaporate the ethanol. The supernatant was then neutralized with 1 M NH_4HCO_3 and lyophilized. The acid/ethanol extract was reconstituted in 0.5 ml of 1 M acetic acid and applied to a 1 x 25 cm column of Sephadex G-50 equilibrated with 1 M acetic acid. Fractions (0.5 ml) were collected at a flow rate of about 10 ml/h and lyophilized. Each fraction was reconstituted in 50 ul of 50 mM Hepes buffer, pH 7.5, and an aliquot (25 ul) was ta ken for the type I IGF receptor kinase assay. The elution position of the IGF-I standard (0.2 ug in 0.5 ml 1M acetic acid containing 0.1% BSA) was determined as described

above.

Protein determination. Protein concentrations were determined by the Bradford method (102) using the Bio-Rad protein dye reagent kit with BSA as the standard. The absorbance measurements were determined on a Gilford Spectrophotometer, model 250.

Statistical analysis. The mean +/- SE was calculated for the cell proliferation assay. Analysis of variance followed by Dunnett's test were performed to determine significant differences between the means; a difference was considered to be significant when $P < 0.05$.

RESULTS

SECTION 1: Effect of IGF-I on the Growth of NSCLC Cells via Activation of the Type I IGF Receptor Tyrosine Kinase

1.1. Type I IGF receptor immunoblot analysis. Expression of the appropriate plasma membrane-associated receptor is required in order for a cell to respond to an extracellular growth factor. All of the NSCLC cell lines examined in this study expressed the type I IGF receptor protein as determined by western blot analysis (Figure 6). Lectin-purified membrane protein (40 ug) from each cell line was subjected to SDS-PAGE, electroblotted to nitrocellulose membranes, then incubated with either a rabbit polyclonal IgG against the alpha subunit of the type I IGF receptor, or a non-specific rabbit IgG. For each cell line, a protein of approximately 132 kD strongly reacted with the antibody directed against the type I receptor. The apparent size of the prominent 132 kD immunoreactive band is similar to the published molecular weight (130-135 kD) of the alpha subunit of the human type I IGF receptor (90).

1.2. Growth effects of IGF-I on lung cancer cells. To determine whether IGF-I could stimulate the proliferation of

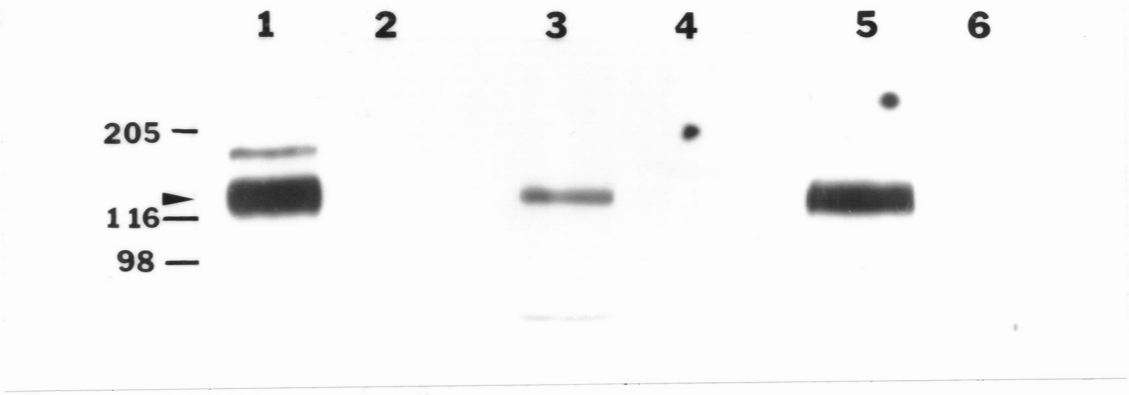

Figure 6. Immunoblot analysis of type I IGF-I receptor expression by lung carcinoma cells. Lectin-purified protein was obtained from each cell line and equal amounts (40 ug) were fractionated by SDS-PAGE (8% gels) in the presence of BME, followed by transfer to nitrocellulose membranes. Membranes were incubated with either a rabbit polyclonal IgG directed against the alpha subunit of the human type I IGF receptor (lanes 1,3,5,7) or a non-specific rabbit IgG (lanes 2,4,6,8). Membranes were washed, incubated with anti-rabbit IgG conjugated to horseradish peroxidase, and bound antibody was visualized by chemi-luminesence. Lanes 1 and 2, protein from A549 cells. Lanes 3 and 4, protein from A427 cells. Lanes 5 and 6, protein from SK-LU-1 cells. The positions of the molecular weight standards are indicated in kilodaltons on the left. **Arrow:** position of the putative 132 kD alpha subunit of the type I IGF receptor.

NSCLC cells, A549, A427, and SK-LU-1 cells were plated at low density (2000 cells/cm^2) and grown for 4 days in the absence or presence of recombinant human IGF-I in serum-free medium (A549, A427) or in the presence of 0.25% FBS (SK-LU-1). The presence of the low concentration of FBS in the SK-LU-1 assay media was necessary to keep the cells attached to the growth surface of the flasks. The rationale for plating the carcinoma cells at low density was that type I IGF receptor numbers decrease as a function of increasing cell density *in vitro* (103). IGF-I was a potent mitogen for the lung cancer cells (Table 1), although the stimulatory response to IGF-I varied among the cell lines. At 5 ng/ml and 25 ng/ml, IGF-I stimulated an increase in A549 cell number of 2- and 3-fold above control, respectively. Likewise, A427 cell number was increased by 1.7- and 2.3-fold above control in the presence of IGF-I, and SK-LU-1 cell number was increased by 1.6-and 2-fold above control after four days in the presence of 0.25% FBS plus IGF-I. These results show that IGF-I promotes the growth of NSCLC cells *in vitro*.

1.3. IGF-I activation of the type I IGF receptor kinase.

1.3.1. IGF-I-stimulated autophosphorylation of the type I receptor. The initial response of the type I receptor to

Table 1

Stimulation of lung carcinoma cell proliferation by IGF-I under serum-free conditions (A549, A427) or in the presence of 0.25% serum (SK-LU-1). The carcinoma cells were plated at 50,000 cells/25 cm^2 T-flask. Cells were grown for 24 h in complete medium, rinsed twice with PBS, then cultured (3 flasks/treatment) in the absence (control) or presence of recombinant IGF-I at the concentrations shown. Media and growth factor were replenished after 48 h, and at 96 h cell numbers were determined by hemacytometer counts.

Cell no.($x10^{-4}$)/Flask[a]

Cell Line	Control	5 ng/ml IGF-I	25 ng/ml IGF-I
A549	12.1 +/- 0.5	25.5 +/- 1.1[b]	35.9 +/- 3.8[b]
SK-LU-1	10.2 +/- 0.5	16.4 +/- 1.0[b]	20.4 +/- 1.5[b]
A427	5.8 +/- 0.3	9.8 +/- 0.6[b]	13.1 +/- 0.4[b]

[a] The data are expressed as the mean +/- standard error of three experiments

[b] $P < 0.05$ with respect to the control value

ligand binding is the autophosphorylation of tyrosine residues located within the tyrosine kinase domain of each beta subunit (95). This allows the type I receptor kinase to phosphorylate intracellular substrates. To demonstrate that the type I IGF receptors expressed by the lung carcinoma cells undergo tyrosine autophosphorylation in response to ligand binding, lectin-purified protein (25 ug) from A549 cells was incubated for 15 min in kinase buffer in the absence or presence of IGF-I as described in Methods. The reaction was terminated by the addition of TCA, and the precipitated proteins were solubilized in Laemmli buffer with and without the addition of BME. The proteins were subjected to SDS-PAGE, electroblotted to nitrocellulose membrane, then incubated with an anti-phosphotyrosine antibody. IGF-I (10 ng/ml and 100 ng/ml) stimulated a dose-dependent increase in the phosphorylation of a single protein with an approximate molecular weight of 98 kD (+ BME) that exists as part of a high molecular weight (> 200 kD, - BME) complex (Figure 7). The size of the phosphorylated protein is similar to the published molecular weight of the type I IGF receptor beta subunit (90). These results indicate that the type I IGF receptor beta subunits undergo ligand-dependent phosphorylation.

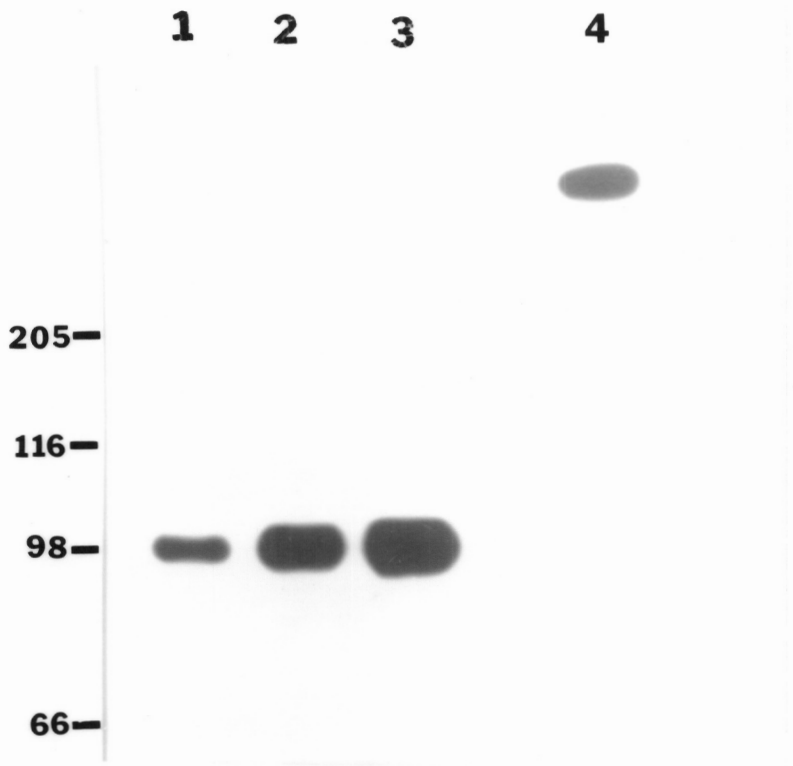

Figure 7. IGF-I-stimulated autophosphorylation of the type I IGF receptor. Lectin-purified protein (25 ug) from A549 cells was incubated for 15 min in 0.1 ml kinase buffer in the presence or absence of IGF-I. The reaction was terminated by the addition of TCA. Precipitated proteins were solubilized in Laemmli buffer with (lanes 1-3) and without (lane 4) beta-mercaptoethanol. Proteins were fractionated by SDS-PAGE (10% gel), transferred to nitrocellulose, then probed with anti-phosphotyrosine antibody. Lane 1, control. Lane 2, 10 ng/ml IGF-I. Lanes 3 and 4, 100 ng/ml. The positions of the molecular weight standards are indicated in kilodaltons on the left. **Arrow:** position of the putative 98 kD beta subunit of the type I IGF receptor.

1.3.2. IGF-I concentration-dependent activation of the type I receptor kinase activity. To demonstrate that autophosphorylation of the type I receptor from lung carcinoma cells activates its tyrosine kinase to utilize exogenous substrates, in vitro phosphorylation of a tyrosine kinase-specific substrate was performed. Lectin-purified membrane protein was obtained from A549 cells and incubated at 25 ug/ml in 72 ul of kinase buffer with or without the addition of IGF-I. After 15 min at 25°C to allow for receptor autophosphorylation (section 3), substrate phosphorylation was initiated by the addition of poly(Glu,Tyr)4:1 (final assay volume of 80 ul). The reaction was terminated 5, 10 and 15 min after the addition of substrate by spotting an aliquot (20 ul) of the assay mixture onto Whatmann 3MM filter paper and washing the filters with acid solution as described in Methods. Filter-bound ^{32}P was determined by liquid scintillation counting. Phosphorylation of poly(Glu,Tyr)4:1 was linear as a function of time between 0-15 min in the presence or absence of IGF-I (Figure 8). IGF-I stimulated a 2- to 5-fold increase in the rate of poly(Glu,Tyr)4:1 phosphorylation relative to phosphorylation of the substrate in the absence of growth factor (Figure 9). These results indicate that IGF-I was able to stimulate the detergent-solubilized type I IGF

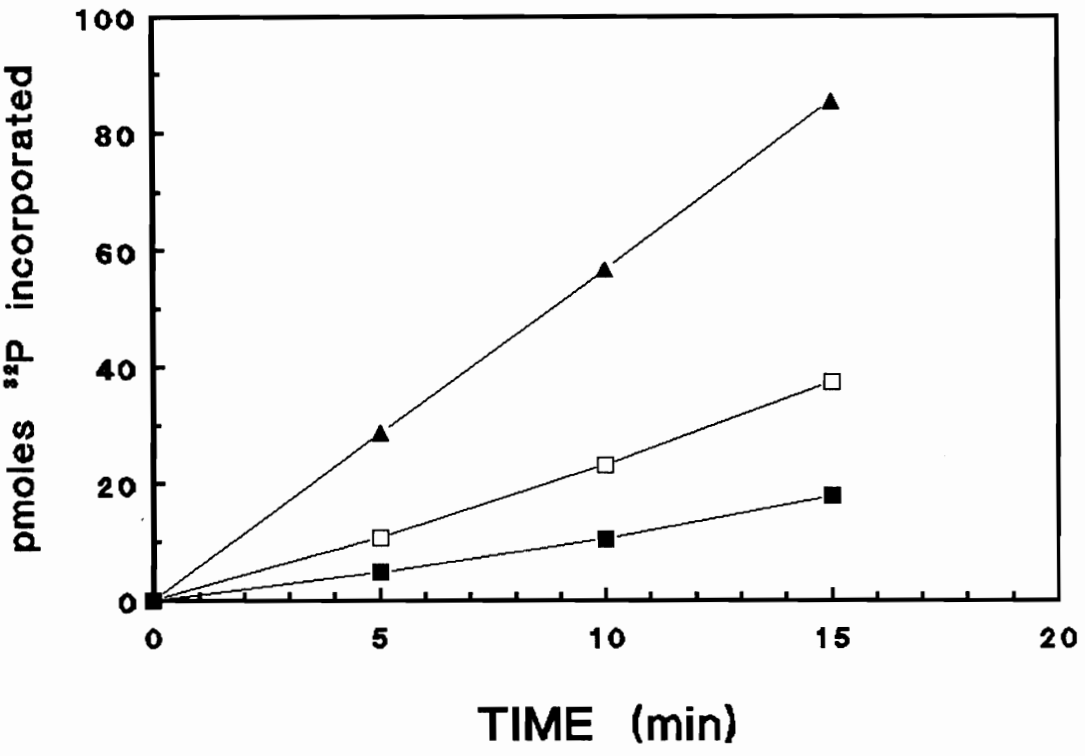

Figure 8. IGF-I stimulation of the type I IGF receptor kinase activity. Lectin-purified protein from A549 cells (25 ug/ml) was incubated for 15 min in kinase buffer containing [gamma ^{32}P]ATP in the absence or presence of various concentrations of IGF-I. Substrate phosphorylation was intiated by the addition of poly(Glu,Tyr)4:1 and the reaction terminated after 5, 10, and 15 min as described in Methods. The data shown is representative of three experiments with different preparations of lectin-purified protein from A549 cells. Shown are only the assays in the absence [■] or presence of 10 ng/ml IGF-I [□] or 100 ng/ml IGF-I [▲].

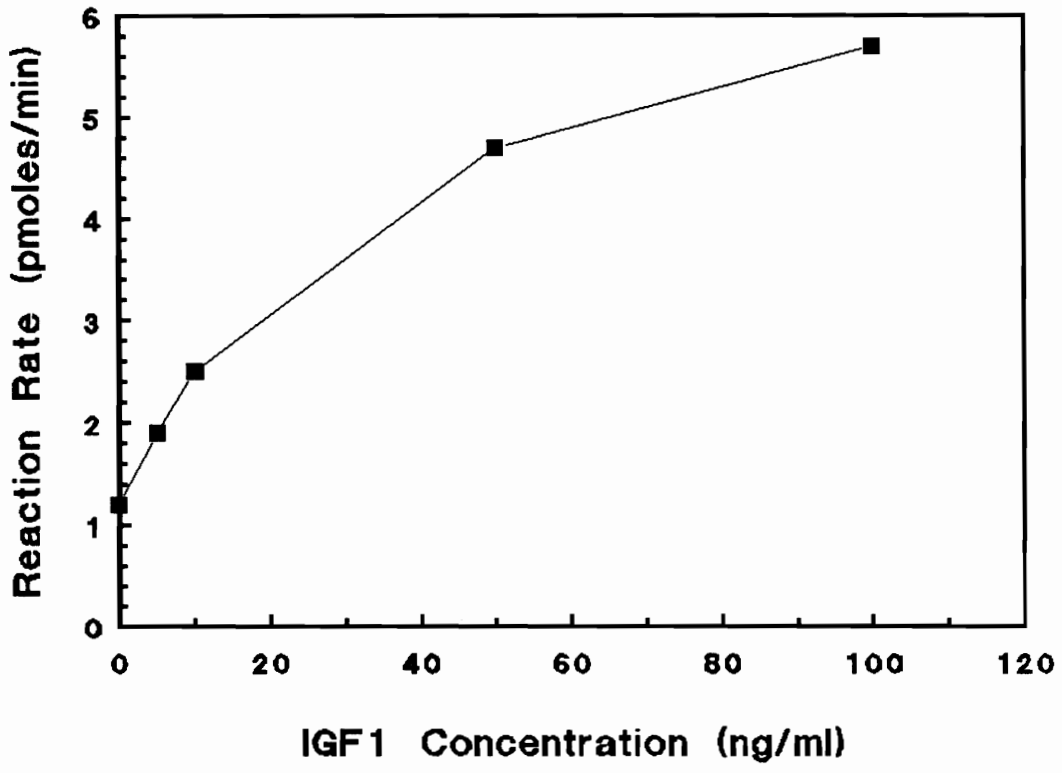

Figure 9. Reaction rate of the ligand-stimulated type I receptor kinase as a function of the IGF-I concentration. Refer to Fig. 8 for details of the experiment.

receptors from A549 cells to phosphorylate an exogenous substrate.

Since IGF-I can bind to the insulin receptor, and thus activate the insulin receptor-associated tyrosine kinase (82), it was determined whether insulin could stimulate the activity of the tyrosine kinase present in the lectin-purified protein preparations from A549 cells. This was done in order to determine that the ligand-activated kinase activity in the lectin-purified protein preparation from the carcinoma cells was due to the activation of type I IGF receptors and not insulin receptors. The reaction was performed as described in Methods, but in the absence and presence of various concentrations of IGF-I or insulin. The reaction was terminated 6 min after the addition of the tyrosine kinase-specific substrate. At the concentrations tested (5 to 100 ng/ml), IGF-I stimulated a dose-dependent 1.5- to 4-fold increase in the phosphorylation of poly(Glu,Tyr)4:1 relative to the phosphorylation of the substrate in the absence of added growth factor (Figure 10). Insulin stimulated a 1.8-fold increase in substrate phosphorylation at the highest growth factor concentration tested (100 ng/ml), but at the lower concentrations (5-50 ng/ml), there was no significant stimulation (< 1.4-fold) of the kinase activity. Because of the lack of a dose-dependent

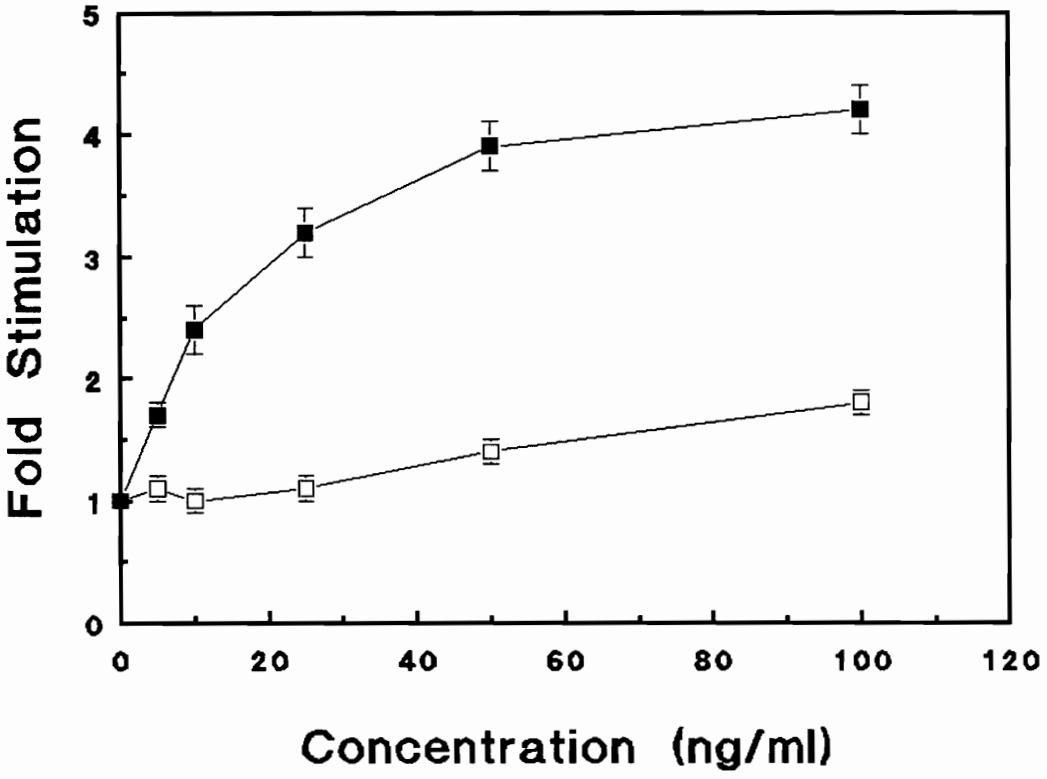

Figure 10. IGF-I and Insulin concentration-dependent stimulation of the type I IGF receptor kinase activity. Lectin-purified protein (25 ug/ml) from A549 cells was incubated for 15 min in kinase buffer containing [gamma-^{32}P]ATP in the absence or presence of either IGF-I [■], or insulin [□], at the concentrations shown. Substrate phosphorylation was initiated by the addition of poly(Glu,Tyr)4:1 and terminated after 5 min as described in Methods. Each point represents the mean +/- standard error of three experiments. Fold stimulation: the incorporation of ^{32}P into substrate in the presence of growth factor/the incorporation of ^{32}P into substrate in the absence of growth factor.

stimulatory effect by insulin, the ligand-activated tyrosine kinase activity in the receptor preparations appears to be solely due to the activation of the type I IGF receptor. The stimulation of the kinase activity by insulin at 100 ng/ml was most likely associated with the type I IGF receptor since insulin has binding affinity for the type I receptor (approximately 20- to 100-fold lower than IGF-I) and thus can activate the receptor-associated tyrosine kinase (82).

1.4. Summary.

The results of the experiments described above indicate that IGF-I is a potent growth stimulatory factor for lung carcinoma cells of NSCLC origin and its activity is mediated through the type I IGF receptor. Carcinoma cell lines derived from NSCLC express the type I IGF receptor (Figure 6). In addition, IGF-I stimulated the autophosphorylation of detergent-solubilized type I IGF receptors from the lung carcinoma cell line A549 (Figure 7) and enhanced the phosphotransferase activity of the type I receptor towards a synthetic tyrosine kinase-specific substrate (Figure 9). Furthermore, IGF-I stimulated the proliferation of each lung carcinoma cell line tested (Table 1).

SECTION 2: Effect of a Lung Fibroblast IGF-I-like Factor on the Growth of NSCLC Cells via Activation of the Type I Receptor Tyrosine Kinase

2.1. Growth effect of fibroblast-conditioned medium on lung carcinoma cells. Human adult lung fibroblasts secrete a factor(s) into their medium that stimulated the serum-free proliferation of A549 cells (Table 2). The LFCM was obtained from CCD-19Lu cultures at population doubling levels between 28 and 30. The protein concentration of the LFCM varied from 5-12 ug/ml. The medium was concentrated by ultrafiltration (3 kd cutoff) and the retentate was further concentrated using Centricon 3 micro-concentrators. For the assay, the retentate was diluted to the desired LFCM protein concentration with SFM/0.1% BSA. After a four day incubation, A549 cell number was increased by 1.5- to 2.1-fold above the control cell number in the presence of 10 ug/ml and 25 ug/ml LFCM protein, respectively. Since the control cell number had increased by 2- to 3-fold above the initial seed number, indicative of autocrine growth, the factor(s) present in the LFCM had stimulated the proliferation of the A549 cells above the level induced by the autocrine growth factors of the cell.

Table 2

Stimulation of lung carcinoma cell proliferation by lung fibroblast-conditioned medium. A549 cells were plated at 20,000 cells/well in six well plates in complete medium. After 24 h, the wells were rinsed twice with PBS then each well received 1 ml of either SFM/0.1% BSA (control) or media supplemented with LFCM protein at 10 or 25 ug/ml (3 wells/ treatment). Media and supplements were replenished after 48 h, and cell numbers were determined after 96 h.

Treatment	Cell no.($x10^{-4}$)/well[a]
Control	5.1 +/- 0.4
10 ug/ml LFCM Protein	7.9 +/- 0.7[b]
25 ug/ml LFCM Protein	10.7 +/- 0.9[b]

[a] The data are expressed as the mean +/- standard error of three experiments

[b] $P < 0.05$ with respect to the control value

2.2. IGF-I immunoblot analysis of fibroblast-conditioned medium. LFCM contained multiple proteins immunologically related to IGF-I. Conditioned media were collected from confluent CCD-19Lu cultures (population doubling level 30) maintained for three consecutive 48 h incubations in SFM. Each LFCM collection was concentrated by ultrafiltration (3 kD cutoff) and the retentate was further concentrated using Centricon 3 micro-concentration units. Equal amounts of protein (100 ug) were subjected to SDS-PAGE in the absence of BME, electroblotted to nitrocellulose membranes, then incubated with either a mouse monoclonal IgG directed against human IGF-I, or a non-specific mouse IgG. Each LFCM collection contained an immunoreactive IGF-I protein of approximately 8 kD (Figure 11). Some LFCM collections contained two immunoreactive IGF-I-like proteins of 8 kD and 13-15 kD (Figure 11, lanes 3 and 7). The immunoblot analysis was repeated several times with different consecutive 48 h collections of LFCM from fibroblast cultures at population doubling levels 28 to 30. All collections contained the immunoreactive 8 kD protein, and some collections contained both the 8 kD and the 13-15 kD immunoreactive protein. In addition, some collections of LFCM contained the IGF-I-like proteins described above and an immunoreactive IGF-I-like protein of 17-20 kD (data not shown). The time of collection

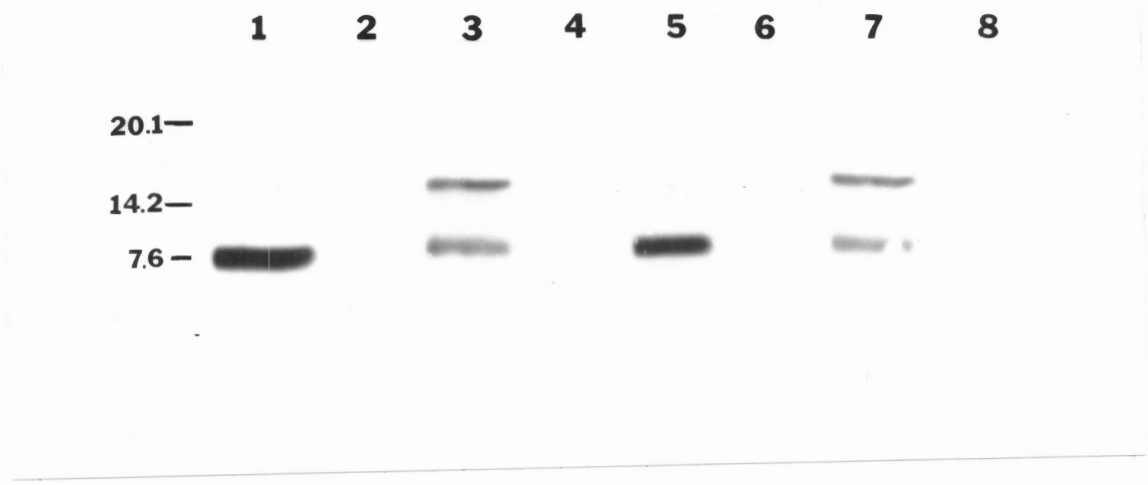

Figure 11. Immunoblot of IGF-I-like factors in lung fibroblast-conditioned medium. LFCM was obtained from confluent cultures of human adult lung fibroblasts as described in Methods. Equal amounts of protein (100 ug) from each LFCM collection were subjected to non-reducing SDS-PAGE (15% gels), transferred to nitrocellulose membranes, then incubated with the anti-IGF-I antibody (lanes 1,3,5,7), or a non-specific mouse IgG (lanes 2,4, 6,8). Blots were incubated with a biotinylated anti-mouse IgG followed by streptavidin-horseradish peroxidase. Bound antibody was detected by chemiluminescence. Lanes 1 and 2, IGF-I standard. Lanes 3 and 4, first 48 h collection of LFCM. Lanes 5 and 6, second 48 h collection of LFCM. Lanes 7 and 8, third 48 h collection of LFCM. The positions of the molecular weight standards are indicated in kilodaltons on the left.

of the LFCM (i.e. first 48 h collection, second 48 h collection, etc..) had no effect on the number of immunoreactive IGF-I-like proteins present in the LFCM. Collections of LFCM from cultures at population doubling levels of 32 to 34 contained only the 8 kD IGF-I-like factor, and immunoreactive IGF-I-like protein was absent, or below detection limits, in LFCM obtained from cultures at population doubling levels higher than 34. Therefore, the presence of the immunoreactive IGF-I-like factors in LFCM appears to be dependent on the population doubling level of the fibroblasts. For collections of LFCM that contained multiple IGF-I-like proteins, the number and size distribution of the immunoreactive IGF-I-like proteins did not change when the proteins were subjected to SDS-PAGE in the presence of the reducing agent BME (5%), although the ability of the anti-IGF-I antibody to detect the reduced IGF-I-like proteins was severely diminished (data not shown). Multiple immunoreactive IGF-I-like proteins were present also in media conditioned for 48 h by cultures of the human fetal lung fibroblast cell line WI-38 (data not shown). These factors were similar in size to the IGF-I-like factors secreted by the adult lung fibroblasts. Immunoreactive IGF-I was not detected in medium conditioned for 48 h by near-confluent cultures of A549 cells.

2.3. Gel filtration of fibroblast-conditioned medium. In order to isolate the immunoreactive IGF-I protein for further studies, LFCM was concentrated by ultrafiltration (3 kd cutoff filter) and the retentate applied to a column (1.5 x 90 cm) of Sephadex G-100 equilibrated with 50 mM HEPES buffer, pH 7.5, containing 50 mM NaCl. Column fractions were assayed for the IGF-I-like protein using the type I IGF receptor kinase assay (Figure 12) and IGF-I immunoblot analysis (Figure 13). The receptor kinase stimulatory factor co-eluted with the immunoreactive IGF-I protein from the column in a single peak. The calculated V_e/V_o value for the receptor kinase stimulatory factor corresponded to an apparent molecular weight of 38 kD. Western blot analysis of the LFCM prior to gel filtration revealed the presence of only the 8 kD immunoreactive IGF-I-like factor (data not shown). This experiment was repeated several times with different collections of LFCM obtained from cultures at population doubling levels of 32 to 34. In each case, the LFCM contained only the 8 kD IGF-I-like protein, but when the LFCM protein was fractionated on the Sephadex G-100 gel filtration column, the immunoreactive IGF-I-like factor co-eluted with the type I receptor kinase stimulatory factor as a single peak with an apparent molecular weight of 35-40 kD. When the Sephadex G-100-purified IGF-I-like protein was

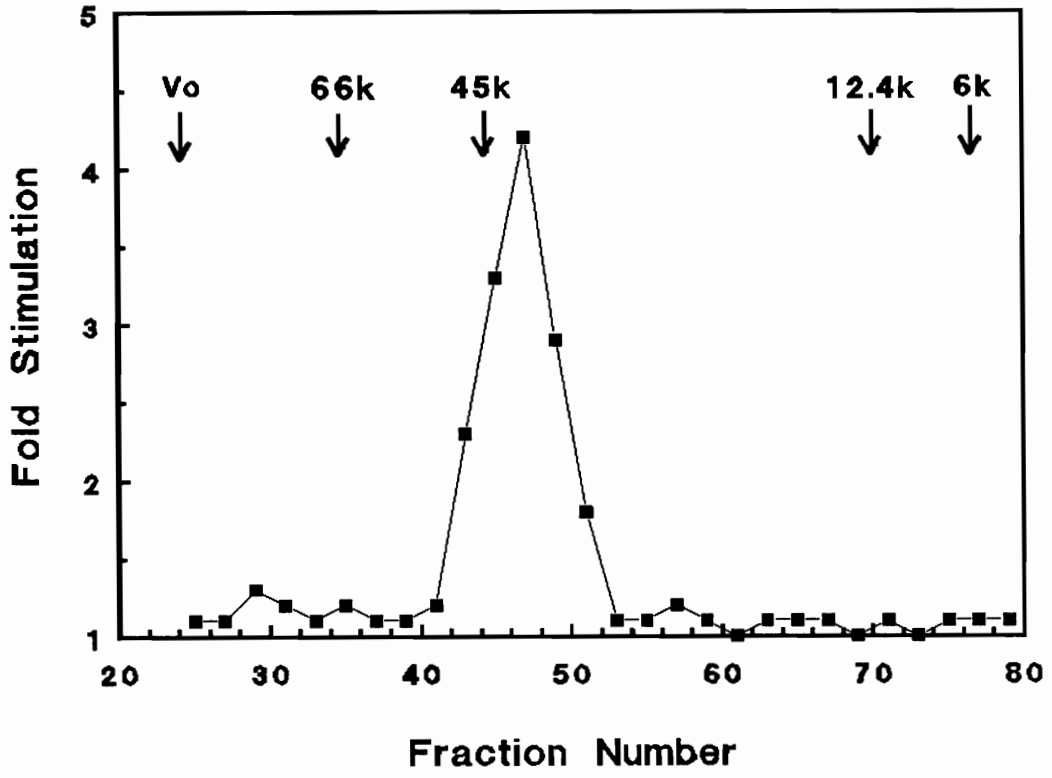

Figure 12. Non-denaturing gel filtration chromatography of the type I IGF receptor kinase stimulatory factor in lung fibroblast-conditioned medium. Concentrated LFCM (2 ml) was applied to a 1.5 x 90 cm column of Sephadex G-100 and the protein eluted in 10 mM HEPES buffer (pH 7.5) containing 50 mM NaCl (18 ml/h). Fractions (1.5 ml) were concentrated and assayed for the ability to stimulate the type I IGF receptor tyrosine kinase from A549 cells as described in Methods. The molecular weight standards used were bovine serum albumin (65 kD), turkey egg albumin (45 kD), horse heart cytochrome c (12.4 kD), and bovine insulin (6 kD). This result is representative of three experiments using different collections of LFCM. Fold stimulation: the incorporation of ^{32}P into substrate in the presence of the column fraction/ the incorporation of ^{32}P into substrate in the absence of the column fraction.

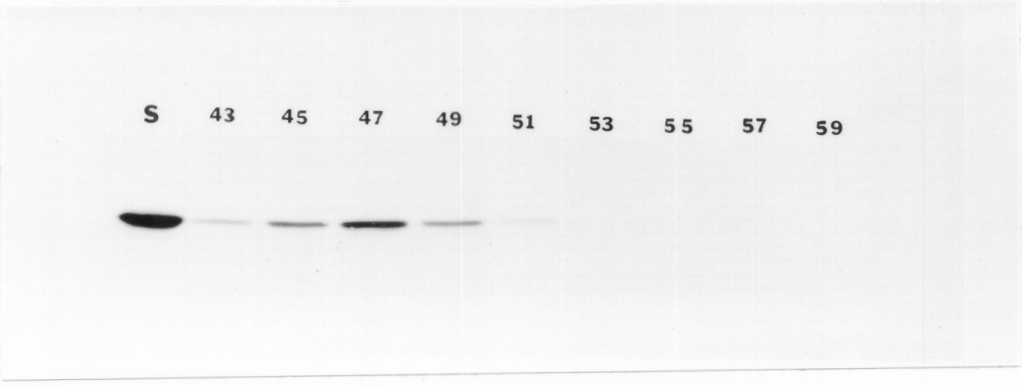

Figure 13. IGF-I immunoblot analysis of fractions from the non-denaturing gel filtration. Refer to Fig. 12 for details of the experiment. Column fractions were assayed for immunoreactive IGF-I as described in Methods. Only the results from fractions 43 to 59 are shown. This result is representative of three experiments using different collections of LFCM. **S**, IGF-I standard.

subjected to acid/ethanol extraction, a procedure which dissociates IGF-I from its binding proteins (101), the IGF-I-like factor nearly co-migrated with the IGF-I standard when chromatographed on a Sephadex G-50 column in 1 M acetic acid (Figure 14). This suggests that the 8 kD IGF-I-like factor in LFCM may be associated with an IGF binding protein.

2.4. Phosphotyrosine immunoblot analysis of the type I IGF receptor following exposure to the fibroblast IGF-I-like factor. To demonstrate that the fibroblast IGF-I-like factor could stimulate the phosphorylation of the type I IGF receptor, lectin-purified protein (25 ug) from A549 cells was incubated for 15 min in kinase buffer in the absence or presence of the Sephadex G-100-purified IGF-I-like factor (final concentrations of 10% and 25% (v/v)) as described in Methods. The reaction was terminated by the addition of TCA, and the precipitated proteins solubilized in Laemmli buffer with and without the addition of BME. The proteins were subjected to SDS-PAGE, electroblotted onto nitrocellulose membrane, then incubated with an anti-phosphotyrosine antibody. The fibroblast IGF-I-like factor stimulated the phosphorylation (Figure 15) of a protein of approximate

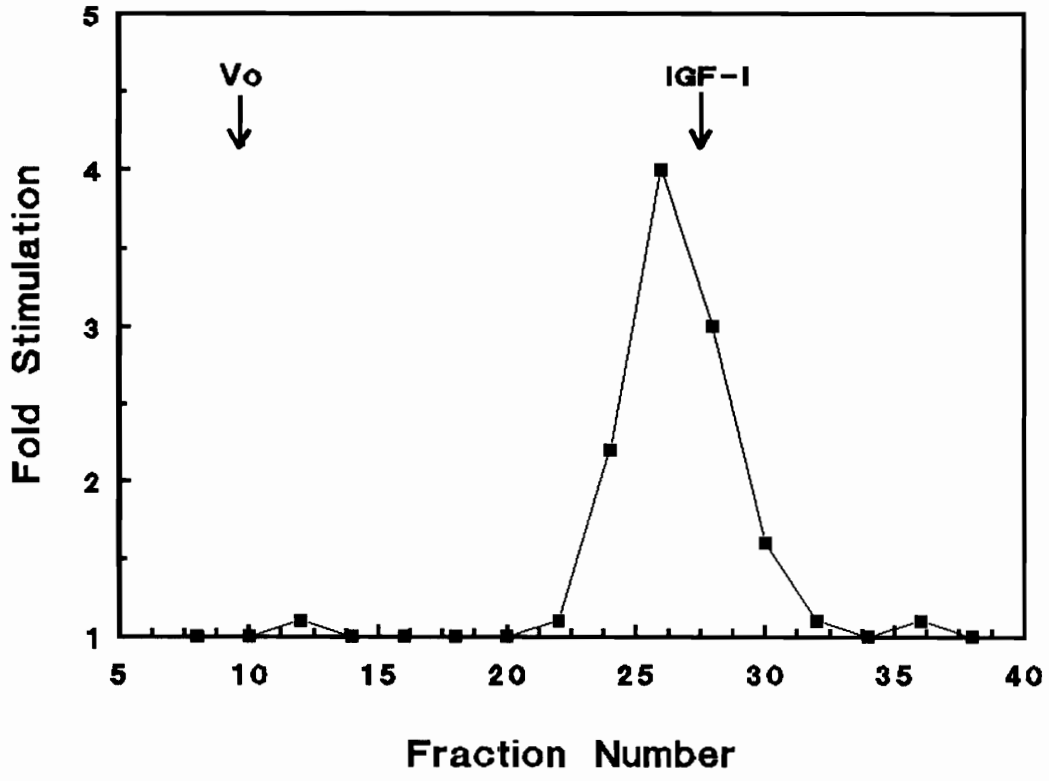

Figure 14. Acid gel filtration chromatography of the acid/ethanol-extracted lung fibroblast-derived IGF-I-like factor. Immunoreactive IGF-I purified by Sephadex G-100 chromatography at neutral pH (Fig. 12) was subjected to acid/ethanol extraction as described in Methods. The lyophilized extract was dissolved in 1 M acetic acid (0.5 ml) and chromatographed on a 1 x 25 cm Sephadex G-50 column in 1 M acetic acid. Fractions (0.5 ml) were lyophilized then reconstituted in 50 ul of 50 mM HEPES buffer (pH 7.5). An aliquot (10 ul) of each reconstituted fraction was assayed for the ability to stimulate the activity of the type I IGF receptor kinase from A549 cells. The column was calibrated using turkey egg albumin (45 kD) for the void volume (Vo) determination, and IGF-I. The experiment was performed twice with similar results. Fold stimulation: refer to the legend to Figure 12.

Figure 15. Stimulation of type I IGF receptor autophosphorylation by the fibroblast IGF-I-like factor. Lectin-purified protein (25 ug) from A549 cells was incubated for 15 min in 0.1 ml kinase buffer in the absence or presence of the IGF-I-like factor purified by non-denaturing gel filtration chromatography (Fig. 12). The reaction was terminated by the addition of TCA. Precipitated proteins were solubilized in Laemmli buffer with (lane 1-3) and without (lane 4) beta-mercaptoethanol. Proteins were subjected to SDS-PAGE (10% gel), transferred to nitrocellulose filter, then incubated with anti-phosphotyrosine antibody. Lane 1, control. Lane 2, 10% (v/v) IGF-I-like factor. Lanes 3 and 4, 25% (v/v) IGF-I-like factor. The positions of the molecular weight standards are indicated in kilodaltons on the left. **Arrow:** position of the putative 98 kD beta subunit of the type I IGF receptor.

molecular weight of 98 kD (+ BME), which exists as part of a higher molecular weight complex (> 205 kD, - BME). A protein of similar size was phosphorylated in the presence of IGF-I (Figure 7). The size of the phosphorylated protein is similar to the published molecular weight of the type I IGF receptor beta subunit (90). These results indicate that the beta subunits of the type I receptor undergo tyrosine phosphorylation in the presence of the fibroblast-derived IGF-I-like factor.

2.5. Fibroblast-derived IGF-I concentration-dependent activation of the type I IGF receptor kinase. The fibroblast IGF-I-like factor stimulated the kinase activity of the type I receptor obtained from different NSCLC cell lines. Lectin-purified protein (25 ug/ml), obtained from A549, A427, and SK-LU-1 cells, was incubated for 15 min in kinase buffer in the absence or presence of various concentrations of the Sephadex G-100-purified IGF-I-like factor. The reaction was terminated 5 min after the addition of the poly(Glu,Tyr)4:1 as described in Methods. Depending on the cell line, the fibroblast-derived IGF-I-like factor caused a dose-dependent 1.6- to 3.6-fold increase in the phosphorylation of poly(Glu,Tyr)4:1 relative to the phosphorylation of substrate in the absence of added growth factor (Figure 16).

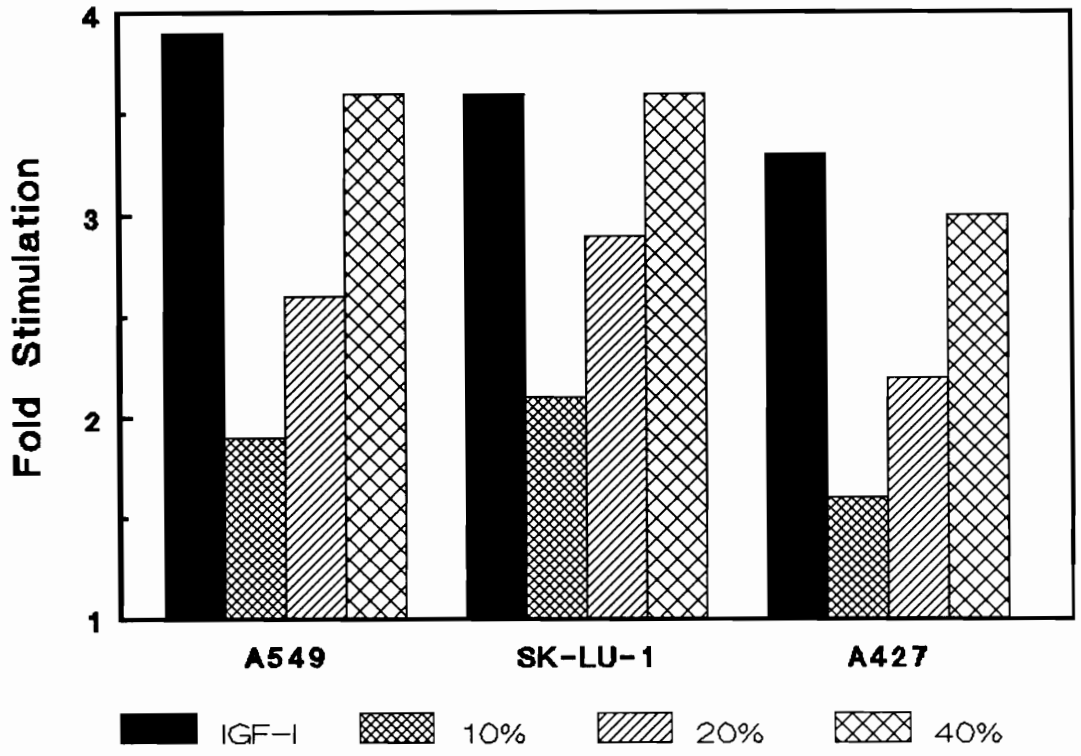

Figure 16. Stimulation of the kinase activity of type I IGF receptors from NSCLC cell lines by the lung fibroblast IGF-I-like factor. Lectin-purified protein (25 ug/ml) from A549, A427, and SK-LU-1 cells were incubated for 15 min in kinase buffer containing [gamma-^{32}P]ATP and various concentrations (v/v %) of the IGF-I-like factor purified by non-denaturing gel filtration chromatography (Fig. 12), or 100 ng/ml IGF-I. Substrate phosphorylation was initiated by the addition of poly(Glu,Tyr)4:1 and the reaction terminated after 5 min as described in Methods. The data are the average of two experiments. Fold stimulation: the incorporation of ^{32}P into substrate in the presence of growth factor/the incorporation of ^{32}P into substrate in the absence of growth factor.

These results suggest that the fibroblast-derived IGF-I-like factor binds to, and activates, type I IGF receptors from NSCLC cells.

Under the conditions of the assay, phosphorylation of poly(Glu,Tyr)4:1 by the type I receptor kinase was linear as a function of time (0 to 15 min) in the presence of the IGF-I-like factor (Figure 17). The reaction was carried out by incubating lectin-purified protein (25 ug/ml), from A549 cells, for 15 min in 72 ul of kinase buffer in the absence or presence of the Sephadex G-100-purified IGF-I-like factor at a concentration of 25% (v/v). Poly(Glu,Tyr)4:1 was added (final assay volume of 80 ul), and the reaction terminated after 5, 10 and 15 min as described in Methods.

2.6. Immunoneutralization experiments. The Sephadex G-100-purified IGF-I-like factor was a potent growth stimulator for the A549 cells. In monolayer growth assays under serum-free conditions, A549 cell number increased about 2-fold above control following a 4 day incubation in the presence of the IGF-I-like factor (Table 3). Pre-incubation of the pooled Sephadex G-100 fractions containing the IGF-I-like factor with an anti-IGF-I antibody (50 ug/ml) completely depleted the growth stimulatory activity. In addition, the

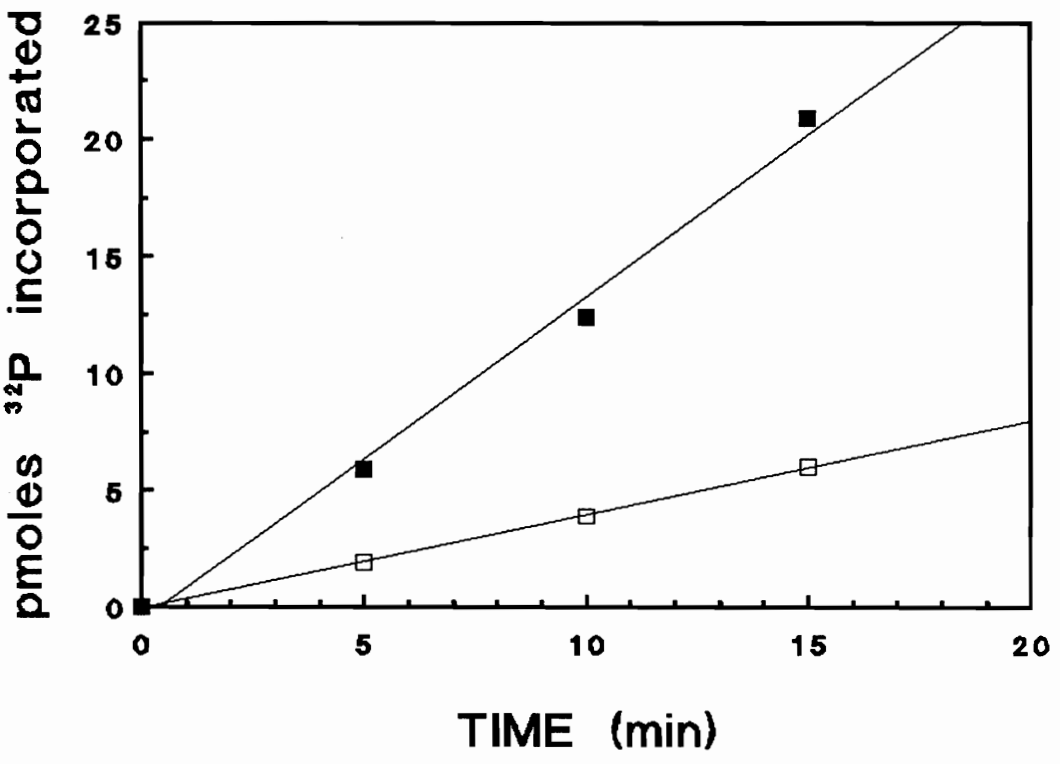

Figure 17. Substrate phosphorylation as a function of time by the fibroblast IGF-I-like factor-stimulated type I IGF receptor kinase. Lectin-purified protein from A549 cells (25 ug/ml) was incubated for 15 min in kinase buffer containing [gamma-^{32}P] ATP in the absence [□] or presence [■] of the IGF-I-like factor (25% v/v) purified by non-denaturing gel filtration chromatography (Fig. 12). Substrate phosphorylation was initiated by the addition of poly(Glu,Tyr)4:1 and the reaction terminated after 5, 10, and 15 min as described in Methods. The data shown is representative of two experiments with different preparations of lectin-purified protein from A549 cells.

Table 3

Neutralization of the growth-promoting and type I IGF receptor kinase stimulatory activities of the fibroblast IGF-I-like factor by the anti-IGF-I antibody. The IGF-I-like factor purified by non-denaturing gel filtration chromatography (Fig. 12) was pre-incubated for 15 min in kinase buffer containing [gamma-^{32}P]ATP and lectin-purified protein (25 ug/ml) from A549 cells in the absence or presence of 25 ug/ml of either the anti-IGF-I antibody or a non-specific mouse IgG. Phosphorylation of poly(Glu,Tyr)4:1 was determined after 5 min as described in Methods. For the monolayer growth assay, A549 cells were grown in six well plates (20,000 cells/well; 3 wells/ treatment) in 1 ml/well of assay media. Control wells received SFM/0.1% BSA. The remaining wells received medium containing the IGF-I-like factor, or medium containing the supernatant from the incubation of the IGF-I-like factor with either the anti-IGF-I antibody or a non-specific mouse IgG as described in Methods. Cultures were replenished with fresh assay media after 48 h, and cell numbers were determined after 96 h.

	Monolayer Growth Assay	**Receptor Kinase Assay**
Treatment	Cell no.(x10^{-4})/well	CPM
Control	5.9 +/- 0.3[a]	8,533 +/- 814[a]
F-IGF-I[b]	12.4 +/- 0.8	27,650 +/- 2,481
F-IGF-I + BPL-M23	5.8 +/- 0.2	8,097 +/- 683
F-IGF-I + non-specific IgG	11.8 +/- 1.0	27,273 +/- 2,759

[a] The data are expressed as the mean +/- standard error of three experiments

[b] Fibroblast IGF-I-like factor purified by non-denaturing gel filtration chromatography

anti-IGF-I antibody (25 ug/ml) completely inhibited the ability of the Sephadex G-100-purified IGF-I-like factor to stimulate the activity of the type I IGF receptor kinase from A549 cells (Table 3). In the absence of antibody, the IGF-I-like factor stimulated a 3-fold increase in the phosphorylation of poly(Glu,Tyr,)4:1. Incubation with a non-specific mouse IgG had no effect on either the growth promoting or the receptor kinase stimulatory activity of the IGF-I-like factor. Coupled with the immunoblot data (Figure 15), these results indicate that the 8 kD IGF-I-like factor was a potent mitogen for the A549 cells and its biological activity was mediated via activation of the type I IGF receptor.

2.7. Summary.

In the experiments described above, it was shown that cell lines of human adult and fetal lung fibroblasts secrete multiple immunoreactive IGF-I-like factors into their medium. The IGF-I-like proteins have apparent molecular weights of 8 kD, 13-15 kD, and 17-20 kD. For the adult lung fibroblasts, the presence of the multiple IGF-I-like factors in the LFCM was dependent upon the population doubling level of the cells. Only the immunoreactive 8 kD IGF-I was detected in the LFCM collections used in the lung carcinoma

growth experiments. The 8 kD IGF-I-like factor stimulated the proliferation of the NSCLC cell line A549. In addition, the IGF-I-like factor stimulated [1] the autophosphorylation of detergent-solubilized type I IGF receptors from A549 cells, and [2] the phosphorylation of a tyrosine kinase-specific substrate by detergent-solubilized type I receptors from all NSCLC cell lines tested. It was determined also that an IGF binding protein may be present in the LFCM which associates with the 8 kD IGF-I-like factor.

SECTION 3: Characterization of the Type I IGF Receptor Kinase Assay

Introduction. In previous sections, it was shown that recombinant IGF-I and the fibroblast IGF-I-like factor stimulate the phosphorylation of poly(Glu,Tyr)4:1 by detergent-solubilized type I IGF receptors from the lung carcinoma cells. In addition, the ligand-stimulated increase in the phosphotransferase activity of detergent-solubilized type I receptors towards the synthetic tyrosine-containing polymer was used to detect the fibroblast-derived IGF-I in fractions obtained from the gel filtration columns. This assay could be potentially useful during the purification of the fibroblast-derived IGF-I. Various parameters of the receptor kinase assay were studied in order to maximize the

sensitivity of the kinase assay for the detection of the IGF-I-like factors. The results are described below.

3.1. Substrate phosphorylation as function of the type I IGF receptor concentration. To demonstrate that the rate of poly(Glu,Tyr)4:1 phosphorylation was dependent on the concentration of the type I receptor in the assay mixture, lectin-purified protein from A549 cells was incubated for 15 min at 25°c in 72 ul of kinase buffer in the absence or presence of 100 ng/ml IGF-I. The concentration of the lectin-purified protein in the reaction mixture was varied from 0 to 50 ug/ml. Substrate phosphorylation was initiated by the addition of poly(Glu,Tyr)4:1 (final reaction volume was 80 ul), and the reaction was terminated after 5, 10, and 15 min by spotting 20 ul of the reaction mixture onto a Whatman 3MM filter then immersing the filter in acid solution as described in Methods. In the absence or presence of IGF-I, the rate of substrate phosphorylation was linear as a function of the concentration of the lectin-purified protein in the reaction mixture within the range of concentrations tested (Figure 18). A difference plot of the reaction velocities (IGF - control) as a function of the lectin-purified protein concentration (Figure 19) indicated that 1.4 pmoles of ^{32}P was incorporated into

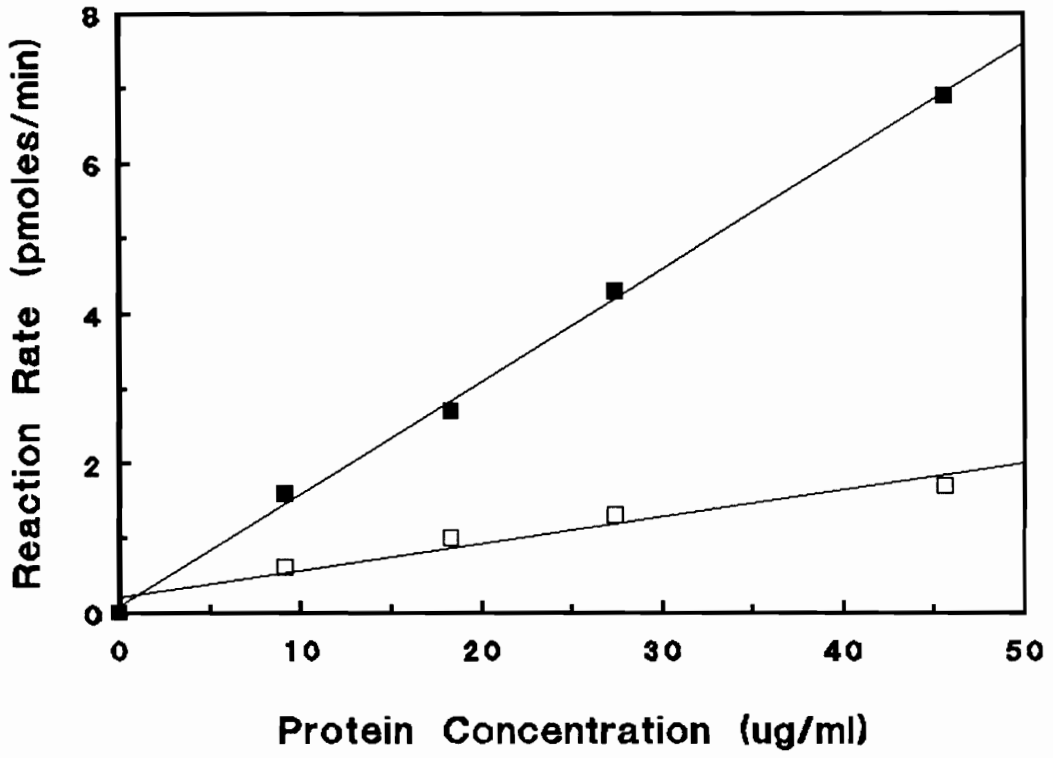

Figure 18. Substrate phosphorylation as a function of the lectin-purified protein concentration from A549 cells. Various concentrations of lectin-purified protein from A549 cells were incubated for 15 min in kinase buffer containing [gamma-^{32}P]ATP in the absence [□] or presence [■] of 100 ng/ml IGF-I. Substrate phosphorylation was initiated by the addition of poly(Glu,Tyr)4:1 and terminated after 5, 10, and 15 min as described in Methods.

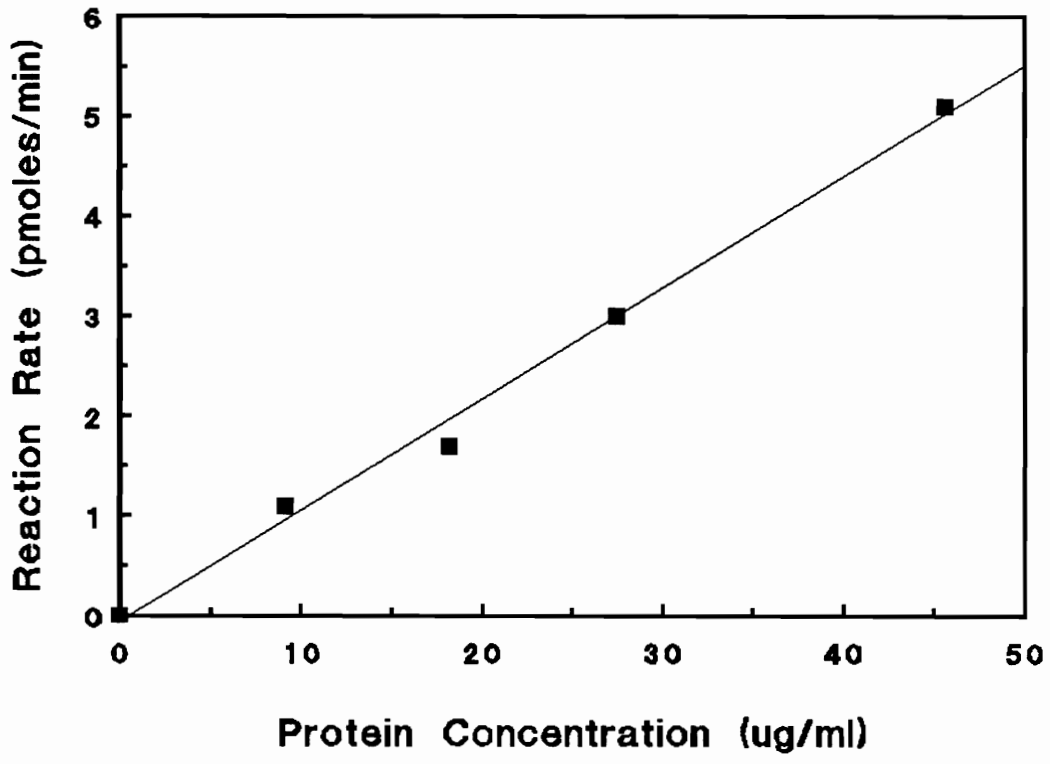

Figure 19. Difference plot (IGF-I stimulated - control) of the reaction rate as a function of the lectin-purified protein concentration from A549 cells. Refer to Fig. 18 for details of the experiment.

poly(Glu,Tyr)4:1/min per ug of lectin-purified protein due to the stimulation of the kinase activity by IGF-I. For multiple preparations of lectin-purified protein from A549 cells, IGF-I (100 ng/ml) stimulated the incorporation of 0.63 - 5.5 nmoles of ^{32}P into poly(Glu,Tyr)4:1/min per mg of lectin-purified protein by the ligand-activated receptor kinase. Therefore, preparations of lectin-purified protein from the A549 cells varied in the specific activity of the IGF-I-stimulated receptor kinase. In general, IGF-I (100 ng/ml) stimulated a 2.5- to 5-fold increase in the rate of phosphorylation of the tyrosine kinase-specific substrate relative to substrate phosphorylation in the absence of growth factor.

Under the conditions of the assay, the phosphorylation of poly(Glu,Tyr)4:1 in the absence of IGF-I may be due to the activity of a tyrosine kinase not associated with the type I receptor. Alternatively, the kinase activity in the absence of IGF-I may be due to the activity of type I receptors that were activated during the course of the purification procedure. A basal level of tyrosine kinase activity in the absence of added ligand is associated also with preparations of the type I IGF receptor isolated from various sources (90,100,104). With respect to the structurally homologous insulin receptor, activation of the

receptor kinase activity by insulin may be due to the binding of ligand to the extracellular alpha subunits which releases the intracellular beta subunits from a conformation which inhibits their intrinsic tyrosine kinase activity (91,92). It is conceivable that binding of the type I IGF receptor (via the highly glycosylated alpha subunits) to the lectin column mimics the binding of IGF-I to the receptor. Various lectins, including wheat germ agglutinin, stimulate the autophosphorylation of EGF receptors (105). As shown in Figure 7, the putative 98 kD beta subunit of the type I IGF receptor was tyrosine phosphorylated following a 15 min incubation in the absence of IGF-I. The immunoblot data supports the argument that the kinase activity in the absence of IGF-I may be due, in part, to activated type I receptors.

3.2. Effect of preincubation time with ATP and IGF-I on the time course of activation of the type I IGF receptor kinase. Autophosphorylation of the type I receptor is required for the activation of the receptor kinase towards the phosphorylation of exogenous substrates. For the conditions of the assay used in this study, the required preincubation time with ATP and IGF-I that would result in the maximum activation of the type I IGF receptor kinase from A549 cells

was determined. The reaction was carried out as described in Methods except that the preincubation time of the lectin-purified protein with ATP (50 uM) in the absence or presence of IGF-I (100 ng/ml) was varied from 0 to 30 min. The reaction was terminated 5 min after the addition of the poly(Glu,Tyr)4:1. Maximum stimulation of the kinase activity by IGF-I with respect to the control kinase activity (fold stimulation) occurred following 10-15 min of preincubation (Figure 20). As little as 5 min of preincubation was sufficient for the near-maximum fold stimulation of the receptor kinase activity by IGF-I. The data described above is similar to the results of Sasaki et al. (100) in which it was shown that maximum autophosphorylation of detergent-solubilized type I receptors from rat liver cells occurs 10 min after the addition of ATP and IGF-I and coincides with the time course of activation of the receptor towards the phosphorylation of poly(Glu,Tyr)4:1.

3.3. Activation of the type I receptor kinase as a function of the Mg^{2+} concentration. It has been determined the divalent cation requirement for the catalytic activity of the type I IGF receptor (100). Of the divalent cations tested (Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+}) only Mg^{2+} and Mn^{2+} are utilized for the kinase activity of the type I receptor.

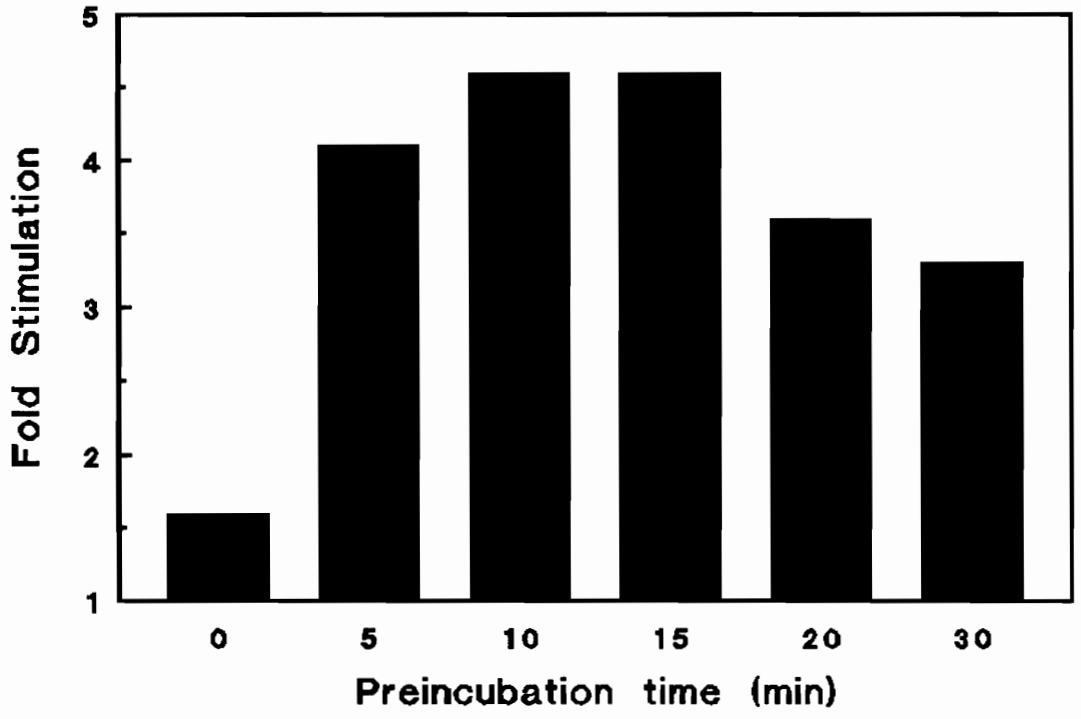

Figure 20. Activation of the type I IGF receptor kinase as a function of the preincubation time with ATP and IGF-I. Lectin-purified protein (25 ug/ml) was incubated for various times in kinase buffer containing [gamma-^{32}P]ATP in the absence or presence of 100 ng/ml IGF-I. Substrate phosphorylation was intiated by the addition of poly(Glu,Tyr)4:1 and terminated after 5 min as described in Methods. The data is representative of three experiments with different preparations of lectin-purified protein from A549 cells. Fold stimulation: the incorporation of ^{32}P into substrate in the presence of growth factor/the incorporation of ^{32}P into substrate in the absence of growth factor.

Mn^{2+} is more effective than Mg^{2+} in promoting the autophosphorylation of the type I receptor whereas Mg^{2+} is more effective than Mn^{2+} in stimulating the phosphorylation of exogenous substrates by the receptor kinase. The biochemical basis for the differential cation preference in the catalytic activities of the type I receptor is not known.

For the assay conditions used in this study, the optimum Mg^{2+} concentration required by the IGF-I-stimulated receptor kinase to promote the phosphorylation of poly(Glu,Tyr)4:1 was determined. The reaction was carried out as described in Methods except that the $MgCl_2$ concentration was varied from 0 to 50 mM. The reaction was terminated 5 min after the addition of poly(Glu,Tyr)4:1. In the presence of IGF-I, 20 mM Mg^{2+} stimulated the maximum incorporation of ^{32}P into poly(Glu,Tyr)4:1 relative to the kinase activity in the absence of growth factor (Figure 21). Stimulation of the receptor kinase activity by IGF-I did not occur when Mn^{2+} served as the cofactor (1 to 20 mM) for the substrate phosphorylation reaction, although the beta subunits of the type I receptor were autophosphorylated as determined by phosphotyrosine immunoblot analysis (data not shown). Furthermore, the receptor kinase activity in the presence of IGF-I (100 ng/ml) and Mg^{2+} (20 mM) was inhibited

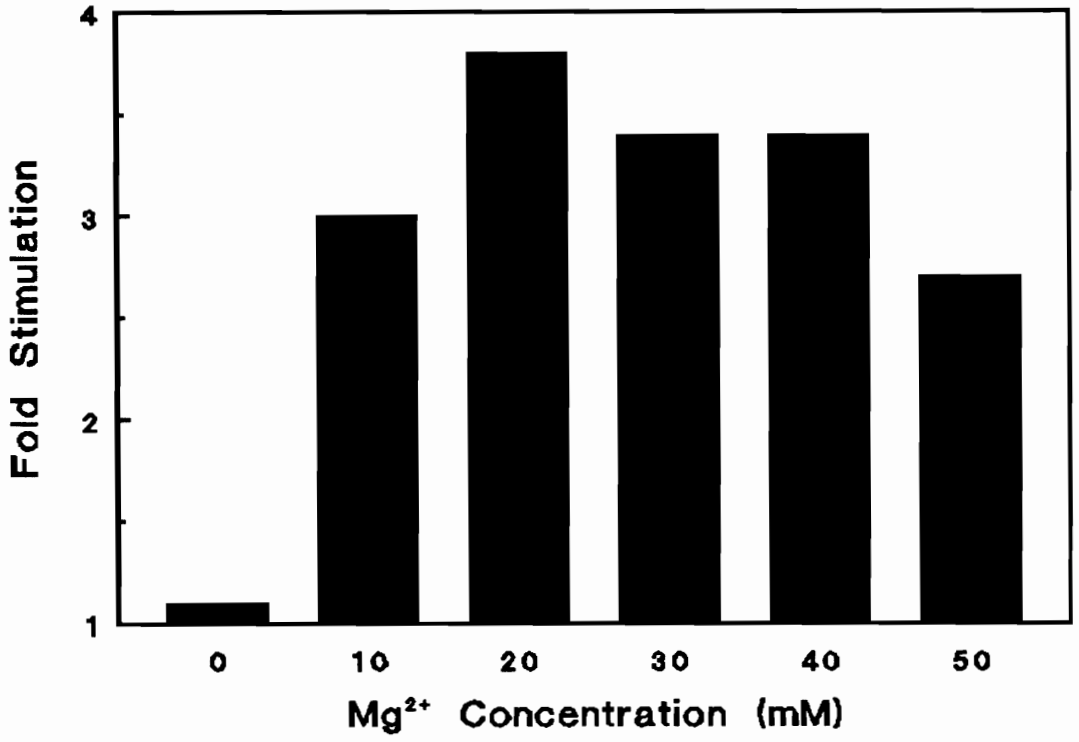

Figure 21. Effect of Mg^{2+} concentration on the type I receptor kinase activity. Lectin-purified protein (25 ug/ml) from A549 cells was incubated for 15 min in kinase buffer containing [gamma-^{32}P] ATP and various concentrations of $MgCl_2$ in the absence or presence of 100 ng/ml IGF-I. Substrate phosphorylation was initiated by the addition of poly(Glu,Tyr)4:1 and terminated after 5 min as described in Methods. The data is representative of two experiments with different preparations of lectin-purified protein from A549 cells. Fold stimulation: refer to the legend to Figure 20.

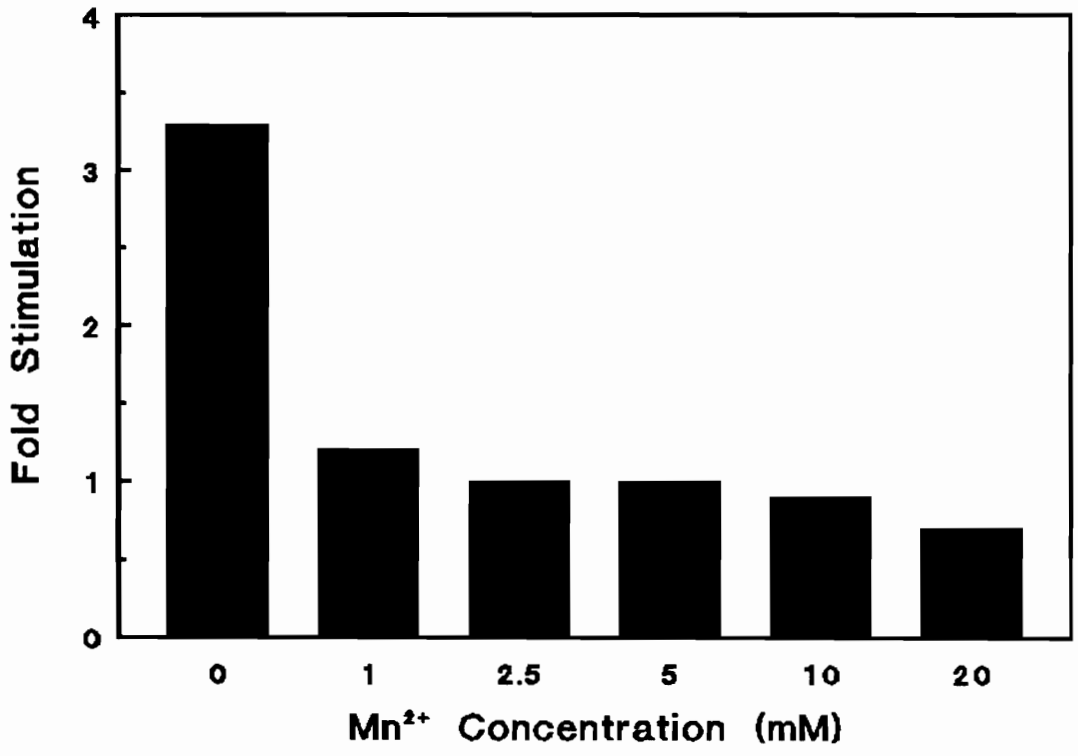

Figure 22. Inhibition of the IGF-I-activated type I receptor kinase activity by Mn^{2+} in the presence of Mg^{2+}. Lectin-purified protein (25 ug/ml) from A549 cells was incubated for 15 min in kinase buffer containing [gamma-^{32}P]ATP, 20 mM $MgCl_2$, and various concentrations of $MnCl_2$, in the absence or presence of 100 ng/ml IGF-I. Substrate phosphorylation was initiated by the addition of poly(Glu,Tyr)4:1 and terminated after 5 min as described in Methods. The data is representative of two experiments with different preparations of lectin-purified protein from A549 cells. Fold stimulation: refer to the legend to Figure 20.

by 90% in the presence of 1 mM $MnCl_2$ (Figure 22). $MnCl_2$ from different manufacturers gave similar inhibitory results (data not shown) which indicate that the inhibition of the kinase activity of the ligand-stimulated type I receptor was not due to an unknown contaminant present in a particular manufacturer's stock preparation of $MnCl_2$.

The reason for the Mn^{2+} inhibition of the IGF-I-stimulated receptor kinase activity is not known. There are no reports in the literature concerning Mn^{2+} inhibition of either the ligand-activated type I IGF or insulin receptors. The use of Mn^{2+} as a cofactor for the receptor kinase in the presence of IGF-I may have resulted in the incomplete autophosphorylation of the three regulatory tyrosine residues located within the catalytic domain of the beta subunit, at positions 1136, 1140, and 1141 using the residue numbering system of Ullrich et al. (81), and thus the receptor kinase could not utilize poly(Glu,Tyr)4:1 as a substrate. Of the three tyrosyl residues in the catalytic domain of the insulin receptor, only the autophosphorylation of Tyr^{1146} is required for insulin stimulation of DNA synthesis (89). In contrast, autophosphorylation of Tyr^{1150} and Tyr^{1151} is required for receptor-mediated insulin stimulation of glycogen synthesis (106).

Alternatively, the use of Mn^{2+} as a cofactor by the IGF-I-stimulated type I receptor kinase could result in the autophosphorylation of tyrosine residues not involved in the regulation of the receptor kinase activity. Autophosphorylation of tyrosine residues located in the carboxyl-terminal tails of the beta subunits of the insulin receptor has no apparent regulatory effect on the kinase activity of the ligand-stimulated receptor (107). The basis for the Mn^{2+} inhibition of the ligand-induced receptor kinase activity was not investigated further.

3.4. Summary.

Some parameters of the type I receptor kinase assay were investigated and optimized:

[1] The rate of substrate phosphorylation was linear as a function of time (0-15 min) and as a function of the lectin-purified protein concentration (0-50 ug/ml) from A549 cells.

[2] Maximum phosphorylation of the exogenous substrate poly(Glu,Tyr)4:1 by the type I receptor required at least 10 min of preincubation of lectin-purified protein with ATP (50 uM), $MgCl_2$ (20 mM) and growth factor. This allowed for the maximum autophosphorylation, and hence activation, of the type I receptor.

DISCUSSION

In order to respond to an extracellular polypeptide growth factor, cells must have the appropriate plasma membrane-associated growth factor receptor. Lung carcinoma cell lines of NSCLC origin express the type I IGF receptor as determined by immunoblot analysis of lectin-purified membrane protein using an antibody specific for the alpha subunit of the type I IGF receptor (Figure 6). Upon binding of IGF-I to its receptor, signal transduction is initiated by the autophosphorylation of tyrosyl residues in the intracellular domains of the receptor by the intrinsic receptor kinase (90,100,104). Autophosphorylation activates the receptor kinase to phosphorylate incompletely-identified intracellular substrates, initiating the cascade of biochemical events that culminates in various biological responses which includes cell proliferation (85,95). In the current study, IGF-I stimulated detergent-solubilized type I receptors from A549 cells to undergo a dose-dependent increase in the autophosphorylation of tyrosyl residues of the beta subunit (Figure 7). In addition, IGF-I stimulated a dose-dependent increase in the phosphorylation of a tyrosine kinase-specific substrate by the solubilized receptor (Figure 9). Coupled with the observation that IGF-I stimulated the monolayer growth of each lung carcinoma cell

line tested (Table 1), it appears that IGF-I binds to, and activates, type I IGF receptors on the lung cancer cells which ultimately results in the stimulation of cell proliferation.

In this study, following the four day assay incubation period in serum-free medium and relative to the other NSCLC cell lines, the A549 cells had not only the greatest growth response to IGF-I (2- to 3-fold above control) but also had the largest increase in cell number in the absence of added growth factor (approximately 3-fold over the initial seed number). As was determined by Siegfried (108), A549 conditioned medium supports the growth of primary cultures of human NSCLC as well as established human lung cancer cell lines. At least one growth factor in the conditioned medium was identified as TGF-alpha (108). IGF-I also was mentioned as a candidate for secretion by A549 cells (109), although no evidence was given for its presence in the conditioned medium. In the present study, immunoreactive IGF-I either was absent or below the limits of sensitivity of our detection system, in medium conditioned for 48 h by near-confluent cultures of A549 cells. The sensitivity of our immunoblot assay was at least 5 ng of recombinant human IGF-I. The results of this study suggests that paracrine-derived IGF-I can stimulate the proliferation of lung carcinoma

cells above the level of growth induced by the autocrine factor(s) of the cell.

However, not all NSCLC cells secrete autocrine growth factors (108). In the present study, A427 cell number increased less than 1.2-fold over the initial seed number after four days in serum-free medium. This indicates that the cells did not synthesize all of the factors necessary for cell proliferation. The growth response of the A427 cells to IGF-I (approximately 2-fold above control) suggests that paracrine-derived IGF-I, either by itself or in conjunction with a competence factor produced by the carcinoma cells, can stimulate cell proliferation. The latter is in keeping with the current view for the requirement of multiple growth factors for the control of cell proliferation (110). Polypeptide growth factors are classified either as competence or progression factors based on which stage of the cell cycle the factors are utilized. Competence factors release cells from a quiescent state (designated as G_0) and allow the cells to enter the G_1 phase of the cell cycle. Progression factors (e.g. IGF-I) allow cells to proceed past various G_1 restriction points and enter S phase.

The data in the present study suggests a potential role for IGF-I in promoting the growth of NSCLC <u>in vivo</u>. Indeed,

it was determined by Minuto et al. (7) that the IGF-I concentration of tissue taken from human primary lung tumors classified as NSCLC is 1.4- to 7-fold greater than the IGF-I content of normal lung tissue taken from the same patient, irrespective of whether the IGF-I concentrations are calculated with respect to tissue weight, hemoglobin content (to normalize differences in the vascularization of neoplastic versus normal lung tissue), or DNA content. In addition, Shigumatsu et al. (96) detected IGF-I and IGF-I binding sites by in situ immunohistochemical and autoradiographic techniques in tissue from resected NSCLC. The specificity of the binding sites for IGF-I relative to IGF-II and insulin was consistant with the binding characteristics of the type I IGF receptor. Furthermore, Reeve et al. (111) detected IGF-I binding proteins in media conditioned by NSCLC cell lines. The binding proteins could be involved in sequestering IGF-I for use by the neoplastic epithelial cells (38).

The lack of IGF-I mRNA expression by NSCLC cells (4) and the absence of immunoreactive IGF-I in media conditioned by some NSCLC cell lines (111,112), suggests that the origin of IGF-I in NSCLC comes from a source other than the neoplastic epithelial cells. As was found in this study, the presence of immunoreactive IGF-I-like protein in media

conditioned by human fetal (WI-38) and adult (CCD-19Lu) lung fibroblasts indicates that the fibrotic stroma of NSCLC may be a source of IGF-I in these tumors. However, it was shown by Minuto et al. (113) that the NSCLC cell line, CaLu-6, releases an IGF-I-like protein into its serum-free medium in increasing concentration as a function of time which parallels an increase in cell number. In addition, CaLu-6 proliferation was inhibited in the presence of an anti-IGF-I antibody indicating that the cell-derived IGF-I-like protein was utilized as an autocrine growth factor. Given the heterogeneity of tumor cell populations, it is likely that some NSCLC cell lines produce IGF-I and others do not. No doubt this may be true for epithelial cell lines derived from a variety of tumors. For example, although IGF-I is considered to be an autocrine growth factor for SCC (112,114,115), not all SCC cell lines secrete IGF-I (112,114,115), nor are IGF-I mRNA transcripts detected in all SCC cell lines (4). In breast cancer, IGF-I mRNA transcripts and/or immunoreactive IGF-I protein have been detected in some breast carcinoma cell lines (101) but not in others (116,117). In the study by Minuto et al. (113), CaLu-6 proliferation was stimulated by the addition of IGF-I suggesting that both autocrine and paracrine-derived IGF-I can promote the growth of the NSCLC cells.

Fibroblast-derived growth factors for lung epithelial cells have been suggested based on the work of numerous investigators. Cultures of HFL-1 human fetal lung fibroblasts secrete a 6 kD factor that stimulates the proliferation of bronchial epithelial cells (118), and the fibroblasts also secrete multiple chemotactic factors for the bronchial epithelial cells (119). Primary cultures of rat fetal lung fibroblasts secrete a growth factor of 30 kD that stimulates the proliferation of type II alveolar cells (120). The secretion of this factor appears to be dependent upon the gestational age of the fetus at the time of the isolation of the lung fibroblasts. In addition, this factor is not secreted by fibroblasts isolated from fetal liver, kidney or skin. In response to glucocorticoids, fetal lung fibroblasts secrete a factor (Fibroblast-pneumonocyte factor) which stimulates surfactant synthesis and secretion by fetal lung type II alveolar cells *in vitro* (121), and enhances fetal lung maturation *in vivo* (122). The lung responds to non-lethal hyperoxic conditions by an increase in the type II cell population as well as an increase in the secretion of pulmonary surfactant by the type II cells (123). The proliferation of type II lung alveolar cells *in vitro* is not stimulated by exposure to hyperoxic conditions. However, in response to hyperoxia, human fetal lung

fibroblasts secrete multiple factors into their medium that stimulate the proliferation and lipid biosynthesis of tumorigenic and non-tumorigenic type II lung alveolar cells.

A paracrine role for IGF-I function in the lung has been suggested in studies of the developing human and rat fetal lung. In situ hybridization and immunocytochemical studies indicate that the mRNAs for the IGFs (IGF-I and IGF-II) in midgestation fetal lung are localized to mesenchymal cells (fibroblasts) but the IGF proteins accumulate in the epithelium (39). In addition, cultures of human (68) and rat (13) fetal lung fibroblasts secrete immunoreactive IGF-I-like protein into their serum-free medium. IGF-I may also play a role in repair processes following insult to the airway epithelium (42). In this context, the IGF-I could be derived from the interstitial fibroblasts and/or alveolar macrophages.

Paracrine interactions between the epithelial and stromal components of a tumor regulate the overall growth of the tumor (124,125), and these interactions may be mediated in part by growth factors (126). Specifically for IGF-I, in situ histo-autoradiographic analysis of resected human breast cancer tissue localized type I IGF receptors to the proliferating epithelial component of the tumors (11), whereas the mRNA transcripts for IGF-I and IGF-II are

expressed by the stromal fibroblasts (10). These results are that consistant with the importance of IGFs as paracrine factors in the growth stimulation of breast cancer epithelial cells.

In the present study, the secretion of multiple immunoreactive IGF-I-like proteins by the adult lung fibroblasts appeared to be dependent on the population doubling level of the cells. Media conditioned by confluent fibroblast cultures at population doubling levels between 28 and 30 contained an immunoreactive IGF-I-like protein with an approximate molecular weight of 8 kD (Figure 11). The majority of the collections of LFCM contained the 8 kD IGF-I-like protein and an immunoreactive IGF-I protein of 13-15 kD. Furthermore, some collections of LFCM contained the two IGF-I-like proteins described above as well as an immunoreactive IGF-I protein of 17-20 kD. Conditioned media obtained from fibroblast cultures at doubling levels of 32 to 34 contained only the 8 kD IGF-I-like factor. Clemmons et al. (128) have shown that several cell culture variables, especially passage number and cell density, regulate the amount of immunoreactive IGF-I produced by cultures of human dermal fibroblasts. However, it was not shown whether these cell culture variables influence the forms of the IGF-I-like factors released into the medium by the fibroblasts.

The high molecular weight (> 8 kD) IGF-I-like proteins produced by the lung fibroblasts could be distinct translational products and thus represent the high molecular weight (13-18 kD) prohormone (IGF-IEa and Eb) forms of IGF-I (63). The expression of the human IGF-I gene results in a primary RNA transcript that undergoes alternative splicing to generate transcripts which encode for prepro-proteins that differ only in their carboxyl-terminal extension region of the E domain (62). Multiple IGF-I mRNA transcripts have been detected in adult rat lung that encode for both prepro-IGF-I proteins (72). Co-translational (amino-terminus) and post-translational (E domain) proteolytic processing of the prepro-proteins generates mature IGF-I (63). The presence of the high M_r IGF-I-like proteins in LFCM could be indicative of the inefficiency in the intracellular (putative site) processing of the prohormones.

Alternatively, the multiple IGF-I-like factors present in the LFCM could represent different stages of an extracellular proteolytic processing of a single translation product (IGF-IEa or Eb) that ultimately generates the 8 kD factor. Many growth factors are synthesized and secreted as inactive pro-proteins that undergo extracellular proteolysis to generate the bioactive polypeptides (129,130).

The secretion of the high M_r IGF-I-like proteins by the lung fibroblasts is not unique to these cells. High molecular weight forms of IGF-I have been detected in the media from a variety of cell lines. A 21.5 kD IGF-I has been purified from media conditioned by human adult dermal fibroblasts (67), a 16 kD IGF-I was isolated from media obtained from the human fetal lung fibroblast cell line WI-38 (68), and a 26 kD IGF-I is secreted by human alveolar macrophages (69). It is likely that these high molecular weight IGF-I-like proteins represent the incompletely processed prohormone form of IGF-I (either Ea or Eb). Furthermore, in each study described above, the high molecular weight IGF-I gave parallel dose-response curves with mature IGF-I for binding to the type I IGF receptor, and stimulated the proliferation of target cells. Thus, there appears to be no difference in the binding affinity of the type I receptor for the different molecular weight forms of IGF-I. With respect to the present work, the results of the above studies suggest that the high molecular weight IGF-I-like protein secreted by the lung fibroblasts are bioactive although only the 8 kD IGF-I-like factor was tested for activity in the cell proliferation and receptor tyrosine kinase assays.

Of particular interest is the secretion of the 16 kD immunoreactive IGF-I by the human fetal lung fibroblast cell line WI-38 (68). In the current study, western blot analysis revealed that media conditioned for 48 h by confluent WI-38 cultures contained multiple IGF-I-like factors similar in mass to the IGF-I-like factors present in the LFCM obtained from the low passage CCD-19Lu cultures. The reason for the discrepency between the studies with respect to the number of IGF-I-like factors present in WI-38 conditioned media is not clear, but could be related to differences in culture conditions and/or differences in the methodology used to detect the immunoreactive IGF-I.

The presence of the IGF-I-like factors in the LFCM was not due to serum contamination: [1] cultures were incubated for two consecutive 48 h periods in serum-free medium and still released multiple IGF-I-like factors into their medium during the course of the third 48 h serum-free incubation, and [2] media obtained from fibroblast cultures at population doubling level 36 did not contain detectable levels of the IGF-I-like factors as assessed by immunoblot analysis.

The immunoreactive IGF-I-like factor produced by the late passage (PDL: 32-34) adult lung fibroblasts had an

apparent molecular weight nearly identical to the IGF-I standard (approximately 8 kD) when analyzed by non-reducing SDS-PAGE, but eluted from the Sephadex G-100 column at neutral pH with an apparent molecular weight of 35-40 kD (Figure 12). Extraction of the Sephadex G-100-purified IGF-I-like factor with acid/ethanol, a procedure that dissociates IGF-I from its binding proteins (101), resulted in the IGF-I-like factor migrating within one fraction of the IGF-I standard on a Sephadex G-50 column equilibrated with 1 M acetic acid (Figure 13). The larger size of the IGF-I-like factor purified by gel filtration at neutral pH could be due to non-specific aggregation of the 8 kD factor which was eliminated under acidic pH conditions. Alternatively, the 8 kD factor could be associated with a binding protein in which the non-covalent interaction between the IGF-I-like factor and the binding protein was stable under the neutral pH condition used for the Sephadex G-100 chromatography but was disrupted when the putative complex was subjected to the acid/ethanol extraction or to the heat treatment in the presence of SDS prior to SDS-PAGE. In vivo, IGF-I is present in extracellular fluids only as a complex with high affinity soluble binding proteins (38). Many cells that produce IGF-I in culture or respond to IGF-I also secrete IGF-I binding proteins, that range in size from

20-50 kD. Although most in vitro studies indicate that the binding proteins function to inhibit the interaction of IGF-I to the type I IGF receptor, it has been reported that human adult dermal fibroblasts secrete a 35 kD binding protein that enhances the binding of IGF-I to the type I receptor (130). Whether the CCD-19Lu cells secrete an IGF-I binding protein was not further investigated, although it has been shown that the 16 kD IGF-I secreted by the fetal human lung fibroblast cell line WI-38 is complexed to a binding protein of 29 kD, and the complex elutes from a gel filtration column under neutral pH conditions with an apparent molecular weight of 45-50 kD (68).

The 8 kD IGF-I-like factor was a potent mitogen for the A549 cells (Table 3). In addition, detergent-solubilized type I IGF receptors from A549 cells underwent tyrosine auto-phosphorylation in the presence of the IGF-I-like factor (Figure 15). Furthermore, when added to lectin-purified membrane protein from each lung carcinoma cell line, the IGF-I-like factor stimulated a concentration-dependent increase in the activity of the type I receptor kinase to phosphorylate an exogenous substrate (Figure 16). Therefore, the lung fibroblast-derived 8 kD IGF-I-like factor appears to be a potent growth stimulator for lung carcinoma cells of NSCLC origin, and its growth promoting

effect is mediated by binding to, and activating, type I IGF receptors.

The potential paracrine secretion of IGF-I by the stroma in NSCLC poses a fundamental question with respect to the regulation of the growth of these tumors in vivo: If the stromal fibroblasts secrete a factor(s) that stimulates the growth of the neoplastic epithelial cells (i.e. IGF-I), can the epithelial cells in turn, secrete a factor(s) that stimulates the growth of the stromal fibroblasts? Although this question was not pursued in the current study, a review of the literature indicates that a paracrine-loop system which involves IGF-I can potentially exist in the lung. Many carcinoma cell lines derived from a variety of tumors, including cell lines derived from NSCLC, secrete PDGF either as the homodimer (a or b chains) or the heterodimer (a and b chains) form, even though the carcinoma cells do not express the PDGF receptor (5). Fibroblasts express the receptor for PDGF (131) and fibroblast proliferation is stimulated by PDGF (132). Furthermore, PDGF stimulates the production/ secretion of IGF-I-like protein by human dermal fibroblasts (127,133) and increases IGF-II mRNA expression by stromal fibroblasts isolated from malignant breast carcinomas (10). Theoretically, the release of PDGF by the neoplastic lung epithelial cells could stimulate the growth of the stromal

fibroblasts and induce the production of IGF-I-like protein by the fibroblasts (Figure 23). The secreted IGF-I in turn can act as a mitogen for the epithelial cells. Of interest is that cell lines derived from SCC, which lack a prominant fibrotic stromata, do not express the gene for PDGF (4). In addition, injection of NSCLC tumor cells into athymic mice results in tumors with an extensive fibrotic stroma, whereas fibrotic stroma was not prominant in tumors formed by SCC cell lines (3). A paracrine-loop signalling pathway between stromal fibroblasts and neoplastic epithelial cells involving IGF-I and PDGF has been proposed to occur in benign and malignant breast carcinomas (10).

In summary, the data presented in this study indicate that IGF-I, and an IGF-I-like factor secreted by human adult lung fibroblasts, are potent mitogens for neoplastic lung epithelial cells of NSCLC origin in vitro. Our data support the hypothesis that the stromal fibroblasts of NSCLC may have a significant role in promoting the growth of the tumor by stimulating the proliferation of the neoplastic epithelial cells via the production of IGF-I-like factors.

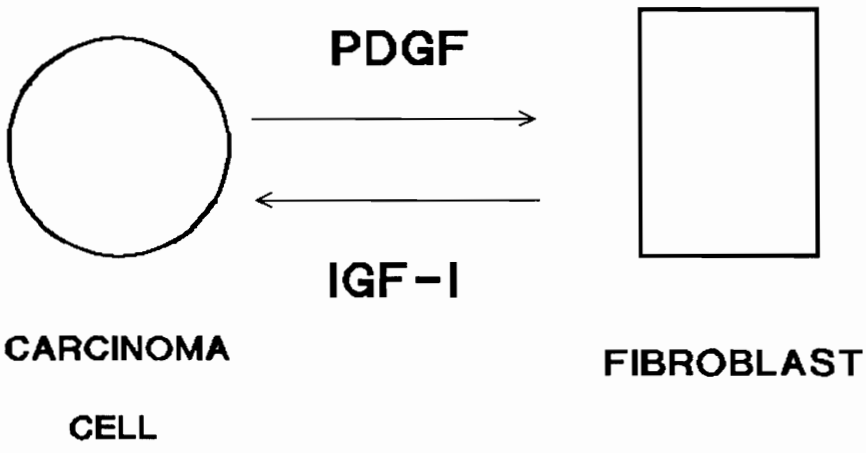

Figure 23. Paracrine-loop signal hypothesis. PDGF secreted by the lung carcinoma cell stimulates [1] the proliferation of the stromal fibroblast and [2] the secretion of IGF-I by the fibroblast. The IGF-I in turn stimulates the proliferation of the lung carcinoma cell.

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VITA

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