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# A mitogenic peptide amide encoded within the E peptide domain of the insulin-like growth factor IB prohormone

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**ABSTRACT** **We have identified an amino acid sequence within the E peptide of the insulin-like growth factor IB (IGF-IB) precursor that is biologically active and designated this peptide insulin-like growth factor IB-(103–124) $E_1$ amide ($IBE_1$). Its existence was predicted by a flanking Gly-Lys-Lys-Lys, a signal sequence for sequential proteolytic cleavage and peptidyl C-terminal amidation. A synthetic analog of the predicted $IBE_1$ peptide, designated Y-23-R-$NH_2$, was generated with tyrosine added at position 0. This peptide at 2–20 nM had growth-promoting effects on both normal and malignant human bronchial epithelial cells. Y-23-R-$NH_2$ bound to specific high-affinity receptors ($K_d = 2.8 \pm 1.4 \times 10^{-11}$ M) present at $1–2 \times 10^4$ binding sites per cell. Ligand binding was not inhibited by recombinant insulin or recombinant IGF-I. Furthermore, a monoclonal antibody antagonist to the IGF-I receptor ($\alpha$IR3) did not suppress the proliferative response induced by Y-23-R-$NH_2$. In addition, C-terminal amidation was shown to be important in receptor recognition since the free-acid analog of $IBE_1$ (Y-23-R-OH) did not effectively compete for binding and was not a potent agonist of proliferation. Immunoblot analysis of human lung tumor cell line extracts using an antibody raised against Y-23-R-$NH_2$ detected a low molecular mass band of $\approx$5 kDa, implying that a protein product is produced that has immunological similarity to $IBE_1$. Extracts of human, mammalian, and avian livers analyzed on an immunoblot with the anti-Y-23-R-$NH_2$ antibody contained proteins of $\approx$21 kDa that were specifically recognized by the antiserum and presumably represent an IGF-I precursor molecule. This implies that in species where an IGF-I mRNA with homology to the human IGF-IB E domain has not yet been described, an alternate mRNA must be produced that contains a sequence similar to that of the midportion of the human IGF-IB E domain. Our findings demonstrate that $IBE_1$ is a growth factor that mediates its effect through a specific high-affinity receptor and is most likely conserved in many species.**

Many peptide hormones originate from precursor molecules that have undergone posttranslational modifications involving several enzymatic events, culminating in the liberation of a mature peptide (1). Peptide amidation is one of the best characterized posttranslational events (2). Amidation is usually initiated at a distinct amino acid consensus sequence consisting of glycine followed by one or more basic amino acids (3). Similar consensus sequences are present in the prohormones of many peptide amide hormones, including gastrin-releasing peptide, substance P, calcitonin, and neuromedin B (4). Prior to the identification of the prohormone motif that signals peptidyl amidation, chemical identification of a peptidyl $\alpha$-carboxyamide had been used to infer biological function in peptides (2). We have identified potential cleavage and amidation sites within one of the prohormones of insulin-like growth factor I (IGF-I) and predicted the resulting peptide amides would be bioactive.

IGF-I has growth-stimulating effects on epithelial and mesenchymal cells *in vivo* (5, 6) and *in vitro* (7, 8) and has been proposed to function as an autocrine or paracrine growth factor in human malignancies including lung tumors (9–13). Developing lung of the human fetus has also been shown to express high levels of IGF-I mRNA (14). IGF-I is produced as a high molecular mass precursor protein that undergoes posttranslational processing to form a 7.6-kDa bioactive molecule (15, 16). The IGF-I gene is single copy and is constructed of five exons that give rise to at least two distinct mRNA products through alternative splicing (17). These two mRNAs, termed IGF-IA and IGF-IB, differ in the 3′ region encoding the E peptide of the prohormone. The IGF-IA mRNA is encoded by exons 1–3 and 5, and the IGF-IB mRNA (Fig. 1*A*) is encoded by exons 1–4 (19, 20). It is thought that the E peptide (Fig. 1*B*) is released from the prohormone during maturation (19).

The predicted proteins resulting from the IGF-IA and IGF-IB mRNAs contain two different C termini (Fig. 1*B*): the IGF-IA mRNA encodes an E peptide of 35 amino acids and the IGF-IB mRNA encodes an E peptide of 77 amino acids containing several potential processing sites (Fig. 1*A*). The existence of these sites led us to postulate that the IGF-IB mRNA could be pluripotential. This is conceptually analogous to the prohormone proopiomelanocortin, which encodes nine hormones (21), the calcitonin gene, which can produce both calcitonin and calcitonin gene-related peptide (22), and the proglucagon mRNAs, which contain three hormones (23). Processing of the IGF-IB E peptide could produce several peptides, including the predicted insulin-like growth factor-IB-(103–124) $E_1$ amide ($IBE_1$), which would be an $\alpha$-carboxyamidated 22-amino acid peptide. We demonstrate here that a synthetic analog of $IBE_1$ functions as a growth factor for both normal and malignant human bronchial epithelial cells. We further demonstrate that a low molecular mass species with immunological similarity to the IGF-IB E domain can be detected in immunoblots from human lung tumor cells and that a large molecular mass species, presumably an IGF-IB E-related precursor molecule, can be detected in extracts from human, mammalian, and avian liver.

## EXPERIMENTAL PROCEDURES

**Cell Culture.** Normal human bronchial epithelial (NBE) cells were obtained from explants of primary tissue as described elsewhere (24). For colony assays, 2000 cells per well were seeded in multiwell dishes as described (24). Data are expressed as percent increase over control colony formation. Primary non-small cell lung carcinoma cell cultures

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Abbreviations: IGF-I, insulin-like growth factor I; $IBE_1$, insulin-like growth factor IB-(103–124) $E_1$ amide; NBE, normal bronchial epithelial; mAb, monoclonal antibody.

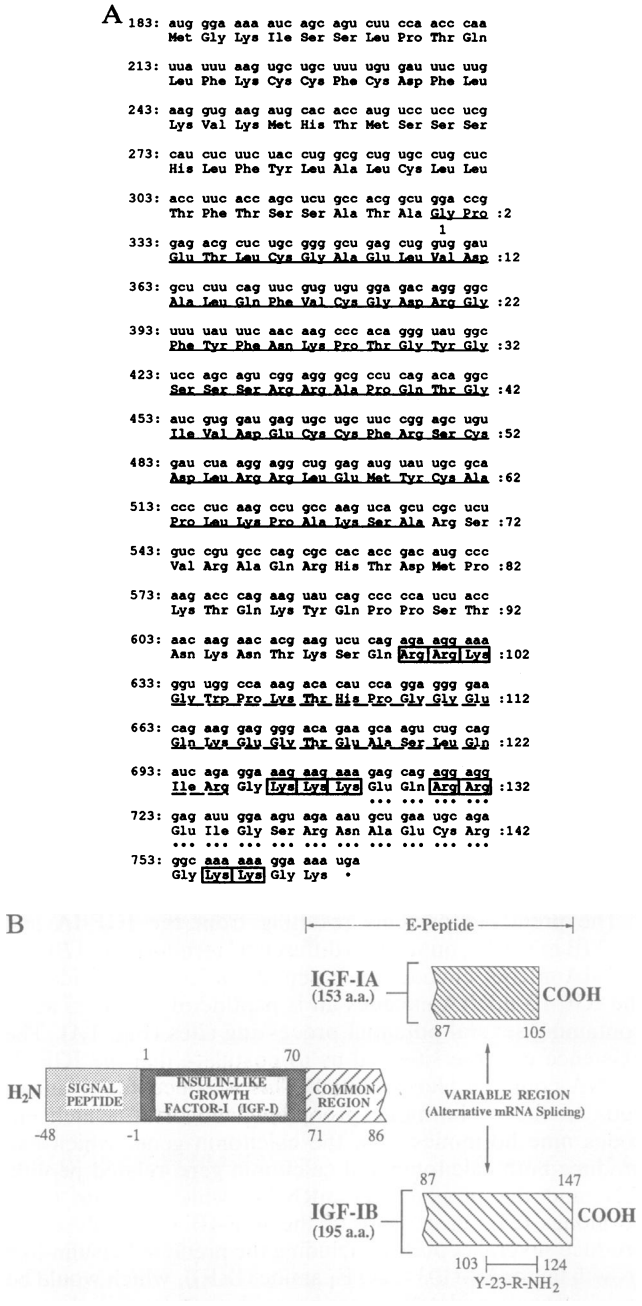


FIG. 1. (*A*) cDNA sequence of the human IGF-IB transcript and the predicted amino acid sequence of its respective precursor molecule, taken from Rotwein (18). Numerical location of base (left) and amino acid (right) sequences are indicated. Solid line identifies the mature IGF-I peptide. Glycine, the first amino acid of mature IGF-I, is noted as position 1. Boxed areas (Arg-Arg-Lys, Lys-Lys-Lys, Arg-Arg, and Lys-Lys) indicate sites of potential proteolytic cleavage. The sequence of $IBE_1$, the proposed peptide amide, is underlined with a dashed line. The predicted processing of this peptide is described in the text. A second potential peptide amide is indicated by the dotted line [IGF-IB-(129–142), $IBE_2$]. (*B*) Illustration of the human IGF-I prohormone showing size and location of the signal peptide (residues −48 to −1), the mature IGF-I peptide (residues 1–70), and the E peptide (residues 71 to the C terminus). PreproIGF-I-(71–86) is common to both prohormones produced by the human IGF-IA and IGF-IB mRNAs. The preproIGF-I peptide containing residues 87 to the C terminus differs in the two prohormones. Y-23-R-$NH_2$ is the tyrosinated synthetic peptide analog of $IBE_1$. aa, Amino acid(s).

were established as described (25) from lung tumors obtained at resection. Colonies of tumor cells that grew were trypsinized and used in colony-forming experiments. Colony assays were performed as described (25). The small cell lung carcinoma cell line NCI-H345 was cultured as described (26). Growth was assessed in semi-solid agarose (26).

To assess effects of the monoclonal antibody (mAb) αIR3 [an IGF-I receptor antagonist (27)], a colony assay was performed using NBE cells in the absence of added insulin or IGF-I. Cells were plated at 1000 cells per ml. mAb αIR3 was used at 1 μg per well, a concentration of antibody that completely blocked IGF-I-induced growth of NBE cells. Recombinant IGF-I or synthetic Y-23-R-$NH_2$ was added with or without mAb αIR3.

**Peptide Synthesis, Iodination, and Binding Assays.** Peptides were synthesized by Applied Biosystems using standard solid-phase methodology and purified to >95% by HPLC. Validation of each synthetic peptide was accomplished by (*i*) total amino acid composition, (*ii*) amino acid sequence, and (*iii*) mass spectrometry. Synthetic peptides were iodinated by the chloramine-T reaction (28) to a specific activity of 27 μCi/μg (1 Ci = 37 GBq). Binding assay was performed at ambient temperature for the times indicated in plastic dishes containing confluent A549 cells. Labeled peptide (0.17 μCi per dish) was added in triplicate in Hanks' balanced salt solution containing 0.1% bovine serum albumin and 50 mM Hepes (pH 7.0). Nonspecific binding was determined by the addition of 10 μg of unlabeled Y-23-R-$NH_2$ (4 μM). Binding was terminated by removing label and washing dishes four times with ice-cold Hanks' balanced salt solution containing 50 mM Hepes (pH 7.0). Cells were solubilized with an overnight trypsin digestion. Cell number per well was determined in triplicate from dishes that underwent the reaction procedure without addition of labeled peptide.

Kinetic parameters were obtained by Scatchard plot, using conditions for the binding assay as described above. Reaction volume was 1.0 ml. Increasing amounts of labeled Y-23-R-$NH_2$ peptide were added in triplicate to obtain a binding curve; a nonspecific control was performed at each concentration of labeled peptide. The programs EBDA and LIGAND were used to obtain kinetic parameters.

**Immunoblot Analysis.** Extracts of cells and livers were analyzed on Western blots (29). For analysis of liver extracts, prepoured polyacrylamide gradient gels were used for protein separations (5–20% acrylamide, purchased from Novex, San Diego), and Tricine was substituted for glycine in the running buffer to resolve low molecular mass proteins. For analysis of cell extracts, polyacrylamide gradient gels were prepared and glycine was used in the running buffer. Immunostaining was performed with rabbit antiserum raised against the synthetic peptide Y-23-R-$NH_2$ (Fig. 1). Commercial acetone liver extracts (Sigma) were used along with detergent lysates from lung tumor cell lines. As a control, antisera was preabsorbed with excess synthetic peptide (2 μM) and immune complexes were removed by centrifugation. Binding of antibody was detected using $^{125}$I-labeled protein A (29).

## RESULTS AND DISCUSSION

**Prediction of the Structure of $IBE_1$.** The structure of $IBE_1$ was proposed by consideration of the predicted amino acid sequence of the IGF-IB prohormone and specificities of posttranslational processing enzymes. The IGF-IB prohormone was selected for this analysis as, unlike the IGF-IA prohormone, the E peptide of IGF-IB contains several regions predicted to be targets of posttranslational processing enzymes that would result in synthesis of a C-terminally amidated peptide. Peptidyl C-terminal amidation is accepted as a signature of hormonal function, and various peptide hormones have been identified based solely on the presence of a C-terminal amidated amino acid (2). The enzymatic processes that transform a prohormone into an amidated peptide generally involve endoproteolytic cleavage, carboxypeptidase trimming of the new C terminus to a glycine-extended peptide, and oxidative conversion of the nitrogen

atom of the C-terminal glycine to an α-carboxyamide. Within the IGF-IB E peptide are several di- and tribasic amino acid regions that are targets of subtilisin-like endopeptidases (30). One region of the IGF-IB E peptide, $Gly^{103}$–$Gly^{125}$, is bracketed by tribasic amino acids (see Fig. 1*A*). Release of the intermediate peptide $Gly^{103}$–$Lys^{128}$ by endopeptidase cleavage C-terminal to the clusters of basic amino acids, followed by carboxypeptidase-H-like removal of the lysines $Lys^{126}$–$Lys^{128}$, would result in a peptide with a C-terminal glycine, $Gly^{103}$–$Gly^{125}$. Both endopeptidases and carboxypeptidase-H-like enzymes have been colocalized to secretory granules (31). The $Gly^{103}$–$Gly^{125}$ peptide would be a substrate for the enzyme complex of peptidylglycine–peptidyl-α-hydroxyglycine monooxygenase and peptidyl-α-hydroxyglycine amidating lyase (32). This enzyme complex converts the nitrogen atom of a C-terminal glycine to the α-carboxyamide of the penultimate amino acid (4). Treston *et al.* (33) have reported that enzymes having peptidylglycine α-amidating activity are present in the secretory granules of neuroendocrine-like cancer cells, including two lines used in this study (NCI-H345 and NCI-N417). The final product of these enzymatic processes would be $IBE_1$. The predicted sequence of $IBE_1$ is GWPKTHPGGEQKEGTEASLQIR-amide.

We searched known peptide sequences for homology to $IBE_1$, using the programs BLASTD and FASTA on the Sun supercomputer at the National Library of Medicine, to the level of 5-amino acid agreement. The human IGF-IB prohormone was found to have 100% homology, as expected. No other protein of human, mammalian, bacterial, or other species origin was found to have >51% homology to the $IBE_1$ sequence. Proteins that were found to have some homology included the *Salmonella* DNA mismatch repair protein MutL (51%) and rat sodium channel cardiac muscle protein (44%). The $IBE_1$ protein can, therefore, be considered unique.

The E domain of the IGF-IB mRNA in rodents, sheep, and other species differs from the human IGF-IB mRNA beginning at nucleotide 585 (34, 35). The known nonhuman forms of the B mRNA also terminate at nucleotide 659. Thus an $IBE_1$-like molecule could not be produced from the known nonhuman B mRNAs. As we show below, analysis of nonhuman liver extracts using an antibody raised against Y-23-R-$NH_2$, containing the sequence presumed to be absent from nonhuman species, detects a 21-kDa protein. This suggests that an mRNA does exist in other species that is homologous to the C-terminal region of the human IGF-IB mRNA and produces an IGF-I-like precursor molecule.

**Activity of a Synthetic Analog of $IBE_1$, Y-23-R-$NH_2$.** We have tested the hypothesis that $IBE_1$ is a bioactive peptide by producing a synthetic peptide derivative of $IBE_1$ with tyrosine added at position 0 to allow radioiodination. We have termed this analog Y-23-R. Two forms of the analog were synthesized, the free-acid derivative Y-23-R-OH and the amidated analog Y-23-R-$NH_2$. The activity of both analogs was examined in a colony-forming assay using NBE cells (Fig. 2*A*). Y-23-R-$NH_2$ produced a concentration-dependent stimulation of cell growth with a maximum effect of 2.3-fold over control growth, whereas the free acid showed little activity. The maximum effect of Y-23-R-OH was a 0.3-fold increase over control at 20 nM. Further increases in concentration of Y-23-R-OH up to 80 nM did not produce any additional effect (data not shown). The activity of Y-23-R-$NH_2$ was also tested using cells derived from primary non-small cell carcinomas (Fig. 2*B*) and a small cell lung carcinoma cell line (Fig. 2*C*). In all cases a growth-promoting effect was observed, with a maximum stimulation between 2.6- and 13.0-fold over control. Maximum stimulation observed with recombinant IGF-I was 3.3-fold (data not shown).

**Specific Binding of Y-23-R-$NH_2$.** Radiolabeled Y-23-R-$NH_2$ peptide was used to assess receptor binding in human lung carcinoma cells that responded to it. Specific binding was found to represent 85% of total binding using excess unlabeled Y-23-R-$NH_2$ in A549 cells (Fig. 3*A*). Specific binding was also found in NCI-H345 cells. Specificity of binding was also determined by comparing the ability of other peptides in excess to compete for radiolabeled Y-23-R-$NH_2$ in NCI-H345 cells. Total binding (6400 ± 1500 cpm) was not inhibited by 2 μM human recombinant insulin (6200 ± 1300 cpm) or 2 μM human recombinant IGF-I (6700 ± 1500 cpm). Y-23-R-$NH_2$ at 2 μM inhibited binding of radiolabel by 75% (1600 ± 200 cpm). The free acid Y-23-R-OH inhibited binding by only 12% (5600 ± 900 cpm). Specific binding was 86% of total in this experiment. We also assessed whether Y-23-R-$NH_2$ could act through the IGF-I receptor. Biological activity of IGF-I and Y-23-R-$NH_2$ was assessed using human NBE cells in the presence and absence of mAb αIR3 (27), which binds to the IGF-I receptor and blocks the effect of IGF-I. As expected, the stimulatory effect of Y-23-R-$NH_2$ was not blocked by mAb αIR3 (Fig. 3*B*). The amidated peptide also did not compete for the binding of radioiodinated IGF-I to its receptor on MCF-7 cells (data not shown).

Scatchard analysis of binding data using the lung carcinoma cell line A549, which is responsive to $IBE_1$, suggests the presence of high-affinity binding sites for Y-23-R-$NH_2$ (Fig. 4). Based on a two-site model of binding, the $K_d$ for the high-affinity site was 28 ± 14 pM. Computer analysis indicated the binding data were consistent either with a two-site model in which a low-affinity site exists with a $K_d$ of 0.49 ± 0.09 μM, or with a model in which there is only one

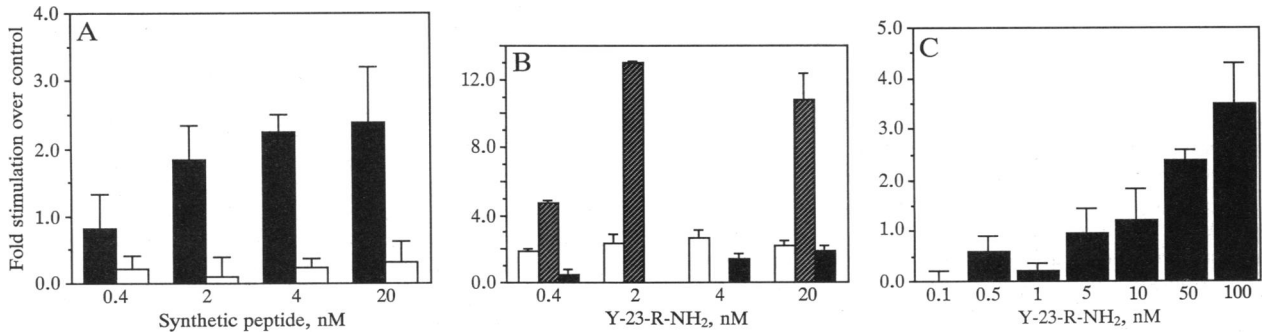


FIG. 2. (*A*) Growth of NBE cells in the presence of increasing concentrations of the synthetic peptide Y-23-R-$NH_2$ (solid bars) or Y-23-R-OH (open bars). NBE cells were obtained from explants of primary bronchial tissue from resection donors. For colony assays, 2000 cells per well were seeded in multiwell dishes in the absence of insulin, IGF-I, or serum as described (24). (*B*) Growth of primary cells from three non-small cell lung carcinomas in the presence of increasing amounts of Y-23-R-$NH_2$. Open bars, response of squamous cell carcinoma (101-87); hatched bars, effect of peptide on large cell carcinoma (105-87); solid bars, effect on adenocarcinoma (114-87). Colony assays were performed as described (25) using 3T3 feeder layers and 1% fetal bovine serum for attachment of primary tumor cells. (*C*) Growth of small cell carcinoma cell line NCI-H345 in semi-solid agarose in the presence of increasing concentrations of Y-23-R-$NH_2$. Assay was performed as described (26).

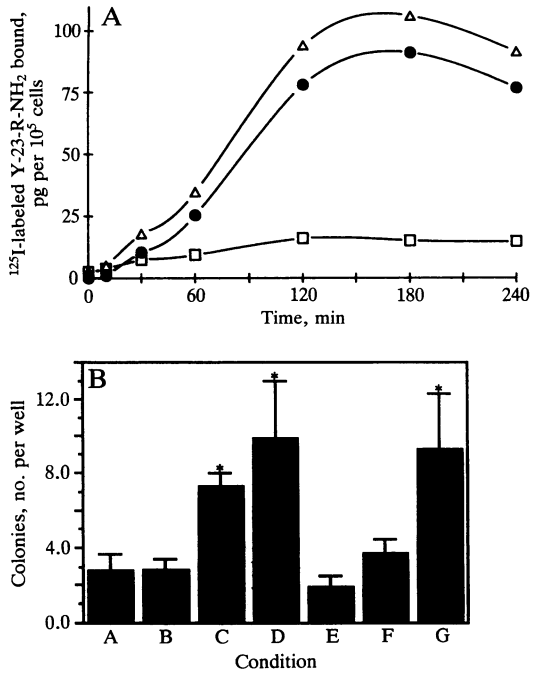


FIG. 3. (*A*) Time course of $^{125}$I-labeled Y-23-R-NH$_2$ binding to the human bronchioloalveolar cell line A549. The peptide ligand was iodinated by the chloramine-T reaction (28) to a specific activity of 27 $\mu$Ci/$\mu$g. Nonspecific binding was determined by the addition of 10 $\mu$g of unlabeled Y-23-R-NH$_2$ to the reaction mixture. Cell number per well was determined in triplicate from dishes that underwent the reaction procedure without addition of labeled peptide. △, Total binding; ●, specific binding; □, nonspecific binding. (*B*) Demonstration that mAb αIR3 (IGF-I receptor antagonist) does not block the proliferative effect induced by Y-23-R-NH$_2$. Graph depicts growth of NBE cells under the following conditions: A, buffer control; B, nonspecific murine IgG control (1 $\mu$g); C, Y-23-R-NH$_2$ (10 ng/ml); D, recombinant IGF-I (10 ng/ml); E, mAb αIR3 (1 $\mu$g) alone; F, mAb αIR3 (1 $\mu$g) plus IGF-I (10 ng/ml); G, mAb αIR3 (1 $\mu$g) plus Y-23-R-NH$_2$ (10 ng/ml). Asterisks indicate significantly different from control at $P < 0.05$.

high-affinity site with a $K_d$ of 33 ± 24 pM, and incomplete inhibition of nonspecific binding was achieved with the concentration of unlabeled competing peptides used. By either model, from the $B_{max}$ of 0.84 ± 0.41 fmol/ml, it was calculated that there are 1–2 × $10^4$ high-affinity binding sites per cell. These binding values determined with the tyrosinated analog Y-23-R-NH$_2$ may differ from those of the presumptive predicted peptide IBE$_1$. The number of high-affinity sites per cell found for Y-23-R-NH$_2$ is in the range known for other amidated peptides, such as gastrin-releasing peptide. Isolated canine antral gastrin cells contain 4 × $10^4$ receptors per cell (36) and guinea pig pancreatic acinar cells contain only 5000 receptors per cell (37), yet both these cell types are target cells for gastrin-releasing peptide.

**Immunoblots of Cell and Tissue Extracts.** To determine whether a small molecular mass peptide with similarity to the putative IBE$_1$ peptide can be detected in cells or tissues, extracts from two lung tumor cell lines and from human and nonhuman liver were analyzed on an immunoblot, using a specific antibody raised against the synthetic peptide Y-23-R-NH$_2$ (Fig. 5). Small cell carcinoma cell lines NCI-N417 and NCI-H345 both showed multiple protein bands that were not present in the lane preabsorbed with excess Y-23-R-NH$_2$ (Fig. 5*A*). Bands were observed at 30, 21, 12, 8, and 5 kDa. The large proteins probably represent precursor forms of IGF-I, transcribed from the IGF-IB mRNA, and the smaller proteins are processed forms. The smallest band may represent native IBE$_1$ or a very similar molecule. Because the

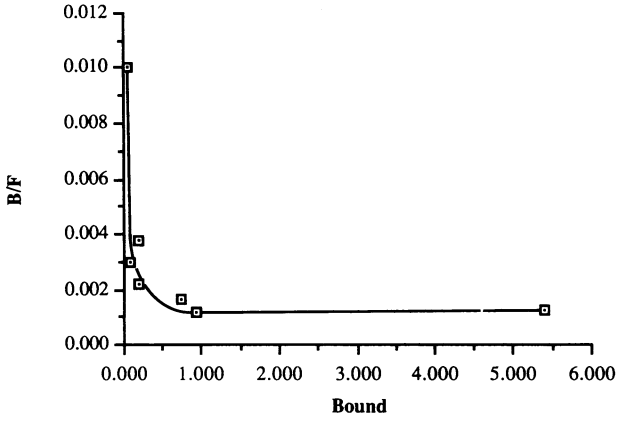


FIG. 4. Scatchard plot analysis of Y-23-R-NH$_2$ binding to target cell A549. Conditions for binding assay were as described in Fig. 3. Reaction volume was 1.0 ml. Increasing amounts of labeled peptide were added to obtain binding curve; a separate nonspecific control was performed at each concentration of labeled peptide. B, bound; F, free. Data for Y-23-R-NH$_2$ are expressed as × $10^{11}$ M.

interspecies conservation of the midportion of the human IGF-IB mRNA was unknown, liver extracts from various species were also examined on an immunoblot to determine whether cross-species expression of an IBE$_1$ epitope could be demonstrated. Fig. 5*B* shows an immunoblot using the Y-23-R-NH$_2$ antiserum. All four species showed a 21-kDa protein reacting specifically with the antiserum, and evidence of some processing in liver was also apparent. A 16-kDa band was observed in all four species, as well as a 6-kDa band in mouse liver. Although a molecule with the expected molecular mass of IBE$_1$ was not observed in liver, it may have been present at a low level not detectable by this technique. These results suggest that even though an mRNA with homology to the E domain of the human IGF-IB mRNA has not yet been described in any other species, an mRNA must exist in other species that is homologous to the proposed IBE$_1$.

**Conclusions.** These data suggest that IBE$_1$ is a growth factor for human lung cells and that the action of IBE$_1$ is mediated by a specific receptor. Thus, more than one peptide product is generated from the IGF-IB prohormone. Since a precursor molecule containing immunoreactivity to the Y-23-R-NH$_2$ peptide was detected in liver extracts from nonhuman species, IBE$_1$ is potentially a growth factor in many species. Preliminary evidence also indicates that a second amidated peptide [IGF-IB-(129–142) E$_2$ amide, designated IBE$_2$, see Fig. 1*A*], predicted from the region adjacent to IBE$_1$, is biologically active. A synthetic analog of this peptide stimulates proliferation of human colon cancer cells (4, 38). The IGF-I gene, like the genes for glucagon (39, 40) and proopiomelanocortin (21), therefore produces multiple peptide products that could have distinct physiological effects in different tissues. Other amidated peptides are known that have multiple functions in different systems. For example, gastrin-releasing peptide is a fetal lung mitogen, a neuroendocrine hormone in the adult gut, and an autocrine growth factor in small cell lung carcinoma (4). It is possible that IBE$_1$ has functions distinct from IGF-I, with distinct organ or cell specificity. It may be fortuitous that IBE$_1$ showed mitogenic activity in the assay system we selected, since our initial prediction was only that it would have some biological activity. Investigation of its properties will be needed to address the question of further biological roles for IBE$_1$.

The approach taken here to identify an alternate peptide product of the IGF-I gene was based upon a prediction from the known sequence of the prohormone. Such a predictive approach has been used by other investigators to identify biologically active peptides. For example, Galindo *et al.* (41)

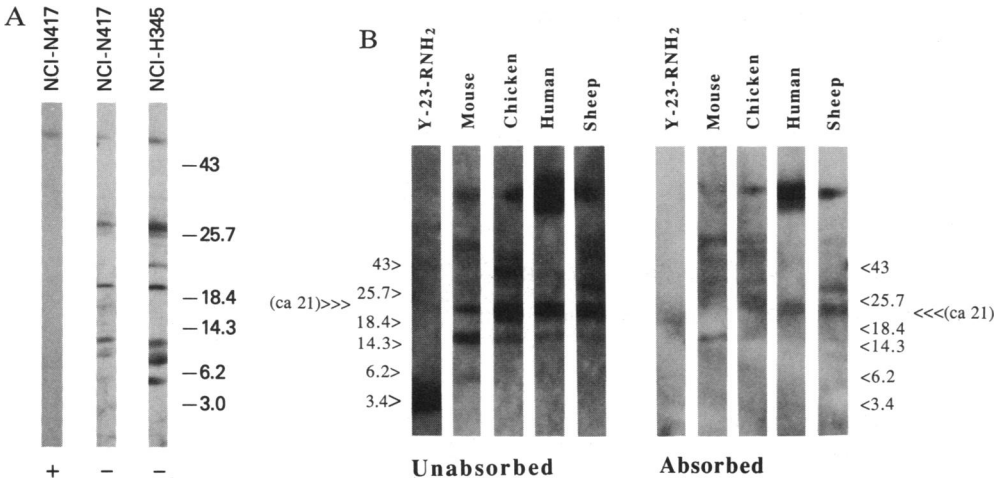


FIG. 5. (*A*) Immunoblot using a polyclonal antiserum against Y-23-R-$NH_2$, of proteins separated by SDS/PAGE from lung carcinoma cell line extracts. Detergent lysates of the cell lines NCI-N417 and NCI-H345 were probed with antiserum alone (lanes −) or antiserum preabsorbed with excess Y-23-R-$NH_2$ (lane +). A 1:1000 dilution of antiserum was used. (*B*) Immunoblot analysis using the same antiserum and conditions as in *A* and proteins from acetone extracts of liver from the four species indicated. Each lane contains 25 μg of protein. Molecular masses in kDa are shown in *A* and *B*.

reported that a 20-amino acid peptide contained within the sequence of chromogranin A was found to inhibit catecholamine synthesis in chromaffin cells. They based the synthesis on the prediction that chromogranin A could be a precursor molecule of peptides that have negative effects on secretion and that proteolytic processing of the precursor released a smaller peptide that is the active species. This is similar to our hypothesis for the release of $IBE_1$ from the IGF-I prohormone, except that here the enzyme activities we posited for production of $IBE_1$ are known to be colocalized to secretory granules along with the prohormone precursor. Using a polyclonal antibody to Y-23-R-$NH_2$, we have identified $IBE_1$-like immunoreactivity in human lung cancer cell lines by immunoblot analysis (Fig. 5*A* and refs. 4 and 42). We have also observed $IBE_1$-like peptides in HPLC fractionation of medium from human lung cancers (data not shown).

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