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Research Article

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Endogenous Insulin-like Growth Factor (IGF) Binding Proteins Cause IGF-I Resistance in Cultured Fibroblasts from a Patient with Short Stature

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Abstract

The ED₅₀ of insulin-like growth factor (IGF)-I-stimulated α -aminoisobutyric acid (AIB) uptake (mean \pm SD) in cultured fibroblasts from a child with short stature that we have reported (1.40 \pm 0.24 nM), is significantly higher than the ED₅₀ of IGF-I-stimulated AIB uptake in fibroblasts from 11 normal subjects (0.42 \pm 0.12 nM) and from 127 short children (0.35 \pm 0.11 nM). Similarly, the ED₅₀ of IGF-I-stimulated thymidine incorporation in fibroblasts from this child is 2.8 times higher than that in fibroblasts from four normal subjects. To minimize potential modulation of IGF-I action by endogenous IGF binding proteins in these assays, fibroblast responsiveness to [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I, an IGF-I variant that has a 600-fold reduced affinity for serum IGF binding proteins, has been examined. The biological activity of this variant is comparable in the patient's and normal fibroblasts, suggesting that the resistance to IGF-I action cannot be attributed to a defective IGF-I receptor. To investigate directly the possibility that IGF-I sensitivity in the patient's fibroblasts is reduced by endogenous IGF binding proteins (IGFBP), binding proteins that are secreted into AIB assay buffer during a 3-h collection and that are cell-associated at the end of the collection have been analyzed. Ligand blot analysis of conditioned AIB assay buffer demonstrates that fibroblasts from the patient secrete 1.3–2.2 times more of *M*_r 46,400/42,900, 32,000, and 26,800 binding proteins than normal fibroblasts. The major difference between fibroblasts from the patient and from normal subjects is a striking 10-fold increase in the amount of a cell surface *M*_r 32,000 binding protein in the patient's fibroblasts. The *M*_r 32,000 binding protein is similar in size to IGFBP-1 and different from IGFBP-2 and IGFBP-3, but it does not cross-react with an antibody against IGFBP-1. We conclude that the resistance to IGF-I action in the patient's fibroblasts is caused by an abnormal production and/or cell association of IGF binding proteins. (*J. Clin. Invest.* 1991. 87:1241–1250.) Key words: insulin-like growth factor • α -aminoisobutyric acid • effective dose

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Introduction

Insulin-like growth factor I (IGF-I)¹ is a major mammalian postnatal growth factor that plays a key role in mediating the growth effects of pituitary growth hormone (1). In responsive cells, IGF-I interacts at the plasma membrane with the IGF-I receptor, a heterotetrameric glycoprotein structurally homologous to the insulin receptor (2). IGF-I binding causes the transmembrane activation of the intrinsic tyrosine kinase activity of the IGF-I receptor. This results, by mechanisms that are not well understood, in rapid insulin-like effects on intermediary metabolism and more slowly developing effects on growth. The response to IGF-I in many tissues may be modulated by high affinity IGF binding proteins (IGFBPs), three of which have been well characterized and termed IGFBP-1, 2, and 3 (3). Several cell types have been shown to produce IGF binding proteins, and these binding proteins may inhibit or enhance IGF-I action (reviewed in reference 4).

The sensitivity of target tissues to IGF-I may be an important determinant of growth. Bierich et al. (5) reported that fibroblasts from a short child with growth failure bound 50% less ¹²⁵I-labeled IGF-I than normal fibroblasts, although the structure and function of the IGF-I receptor in this child's fibroblasts were not directly examined. We have investigated IGF-I sensitivity in cultured skin fibroblasts from patients with short stature and have reported that fibroblasts from one child were significantly less responsive to stimulation by IGF-I in α -aminoisobutyric acid (AIB) uptake assays (6). The ED₅₀s of IGF-I-stimulated AIB uptake (mean \pm SD) in fibroblasts from 11 normal subjects (0.42 \pm 0.12 nM) and from 127 short children (0.35 \pm 0.11 nM) are similar (Heath-Monnig, E., D. P. Dempsher, and W. H. Daughaday, unpublished results) and significantly lower than the ED₅₀ of IGF-I-stimulated AIB uptake in fibroblasts from this patient (1.40 \pm 0.24 nM). IGF-I binding to the patient's fibroblasts suspended by brief treatment with trypsin appeared to be normal, although interpretation of these studies was complicated since treatment of the cells with trypsin could potentially modify the IGF-I receptor and would remove adherent binding proteins (7, 8).

In this study, we have used an IGF-I variant that interacts minimally with IGF binding proteins to examine fibroblast responsiveness. This variant, in which residues 3, 4, 15, and 16 have been changed from G³,T⁴,Q¹⁵,F¹⁶ to Q³,A⁴,Y¹⁵,L¹⁶, has a 600-fold reduced affinity for serum binding proteins but an affinity for the IGF-I receptor that is equal to that of IGF-I (9). The comparable biological activity of this IGF-I variant in nor-

1. Abbreviations used in this paper: AIB, α -aminoisobutyric acid; CSC, child with short stature; IGF, insulin-like growth factor; IGFBP-1,2,3, IGF binding protein 1,2,3.

mal and the patient's fibroblasts suggested that the resistance to IGF-I action in the patient's fibroblasts could not be attributed to a defective IGF-I receptor. We report here that IGF-I sensitivity in this patient's fibroblasts is reduced by an abnormal production of IGF binding proteins.

Methods

Materials. AIB, Hepes, polyethylene glycol (PEG) 8000, BSA, bovine gamma-globulins, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, *N,N*-dimethylformamide, L-glutamine, and RPMI 1640 medium were purchased from Sigma Chemical Co., St. Louis, MO. Disuccinimidyl suberate was obtained from Pierce Chemical Co., Rockford, IL. Alkaline phosphatase-conjugated goat anti-rabbit IgG was supplied by Calbiochem-Behring Corp., San Diego, CA. α -[Methyl-³H]-aminoisobutyric acid ([³H]AIB), 33.5 Ci/mmol, and [methyl-³H]thymidine, 20 Ci/mmol, were purchased from DuPont/New England Nuclear Research Products, Boston, MA, and Na¹²⁵I, 13–17 mCi ¹²⁵I/ μ g I, was purchased from Amersham Corp., Arlington Heights, IL. FCS, DME, and MEM were routinely supplied by Gibco Laboratories, Grand Island, NY, and FCS was also obtained from J. R. Scientific, Inc., Woodland, CA. Tissue culture supplies were obtained from the following companies: 100-mm culture plates from Corning Glass Works, Corning, NY, 150-mm culture plates from Falcon Plastics, Lincoln Park, NJ, and 24-well tissue culture clusters from Costar, Cambridge, MA. Nitrocellulose sheets were obtained from Schleicher and Schuell, Inc., Keene, NH.

Recombinant human [T⁵⁹]IGF-I was obtained from AmGen Biologicals, Thousand Oaks, CA, and was used as native IGF-I in these studies. [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I was prepared by site-directed mutagenesis of a synthetic gene as previously described (9). ¹²⁵I-labeled IGF-I (0.80–1.42 Ci/ μ mol) and ¹²⁵I-labeled [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I (0.78–1.22 Ci/ μ mol) were prepared as previously described (10).

Ascites fluid containing the MAb to the IGF-I receptor, α IR-3 (11), was kindly provided by Dr. Steven Jacobs (The Wellcome Research Laboratories, Research Triangle Park, NC). α IR-3 was affinity purified from the ascites fluid to remove IGF binding proteins using an Econo-Pac Protein A Kit, essentially as described by the manufacturer (Bio-Rad Laboratories, Richmond, CA). Rabbit anti-human IGFBP-1 antibody, raised against purified amniotic fluid IGFBP-1, and rabbit anti-human IGFBP-3 antibody (12), were kindly provided by Dr. David Clemmons (University of North Carolina School of Medicine, Chapel Hill, NC) and by Dr. Robert Baxter (Royal Prince Alfred Hospital, Camperdown, NSW, Australia), respectively.

Cell culture. The following skin fibroblasts from three normal subjects were obtained from the Human Genetic Mutant Cell Repository, Coriell Institute of Medical Research, Camden, NJ: GM3349 from a 10-yr-old boy, GM5565 from a 3-yr-old boy, and GM5757 from a 7-yr-old boy. Fibroblasts from two other normal subjects, an 18-yr-old man (WUMS1) and a 37-yr-old man (WUMS2), were isolated in our institution. Fibroblasts from the patient, which we have designated CSC, were obtained at 9.8 yr of age, because her serum IGF-I level was elevated/high normal when measured by two different methods. Clinical data on the patient (Patient 1) have been presented previously (6). From 13 to 15.5 yr of age, the girl received recombinant human growth hormone (0.3 mg/kg/wk). She achieved a final height of 156 cm (Dr. Hulda Wohltmann, Charleston, SC, personal communication).

Fibroblast stock cultures were maintained in DME containing antibiotics (0.625 μ g/ml amphotericin B, 100 μ g/ml penicillin G, 100 μ g/ml streptomycin sulfate) and 15% FCS, as previously described (6). Experiments were performed on fibroblasts at passages 6–13, plated in DME containing antibiotics and 10% FCS. All the cultures were fed 3–4 d after plating. The cells were studied at confluency 6–9 d after plating.

The human hepatoma cell line HepG2, which produces IGFBP-1 (13), and the human T helper cell line Jurkat, which produces IGFBP-2 (14), were obtained from Dr. Richard Ostlund and Dr. Dennis Loh,

respectively, at this institution. HepG2 cells were maintained in MEM containing antibiotics, 4 mM L-glutamine, and 10% FCS. Jurkat cells were maintained in MEM with 25 mM Hepes/RPMI 1640 medium (1:1) containing antibiotics, 2 mM L-glutamine, and 10% FCS.

AIB uptake studies. AIB uptake assays were performed in 24-well clusters as previously described (6), using an assay volume of 250 or 500 μ l as indicated. Day 8 cell monolayers were used.

Thymidine incorporation studies. Thymidine incorporation assays were performed in 24-well clusters as described by Sasaoka et al. (15) with minor modifications. Briefly, day 8 cell monolayers were washed three times with Puck's Saline G and then incubated with increasing amounts of IGF-I or [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I in serum-free medium (MEM with 25 mM Hepes, pH 7.4, antibiotics, and 1 mg/ml BSA) in a volume of 500 μ l. After a 20-h incubation at 37°C in room air, [³H]-thymidine (final concentration, 0.5 μ Ci/ml) was added for 4 h. The cells were then washed with ice-cold Puck's Saline G three times, 10% TCA twice, 95% ethanol/ether (3:1) twice, and air dried. TCA-precipitable material was dissolved in 500 μ l of 1% SDS, and a 200- μ l aliquot was counted in a Packard Tri-Carb 1500 liquid scintillation counter. The ED₅₀ for IGF-I or [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I stimulation of thymidine incorporation was determined exactly as described for AIB uptake (6).

IGF-I binding to fibroblast monolayers. IGF-I binding studies were performed in 24-well clusters as previously described (16). Briefly, day 9 cell monolayers were incubated at 4°C with ¹²⁵I-labeled IGF-I or ¹²⁵I-labeled [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I and either increasing amounts of unlabeled ligand or 2.3 μ g/ml α IR-3 in serum-free medium. The final volume was 250 μ l. After an 18–20-h incubation, the cells were washed three times and then solubilized in 1% SDS. An aliquot was counted in a Packard Auto-gamma 500C gamma counter. In preliminary experiments, we found that IGF-I and [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I binding to both normal and CSC fibroblasts reached a steady state at 18 h. Cell counts were determined from replicate wells in each experiment.

Affinity cross-linking analysis. Day 6 or 8 cell monolayers were washed three times with serum-free DME separated by 20-min incubations at 37°C, and affinity cross-linking analysis was performed as previously described (17).

Collection of fibroblast binding proteins. 0.5 \times 10⁶ cells were plated onto 100-mm plates in 10 ml DME containing antibiotics and 10% FCS in order to scale-up the incubation conditions used in the AIB uptake assay. Day 8 cell monolayers, untreated or pretreated for 2 d with serum-free medium at 37°C in room air, were used. The serum-free medium was collected, clarified by low speed centrifugation, and stored at –20°C until assayed. The monolayers were washed three times with Krebs-Ringer phosphate buffer, pH 7.37, with 5.5 mM glucose. 6.55 ml of AIB assay buffer (Krebs-Ringer phosphate buffer, pH 7.37, with 5.5 mM glucose and 1 mg/ml BSA) was then added (corresponding to a 250- μ l assay volume). After a 3 h incubation at 37°C in room air, the conditioned AIB assay buffer was removed, clarified by low speed centrifugation, and stored at –20°C until assayed. The cells were washed three times with Puck's Saline G and were solubilized in 500 μ l of electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue) without 2-mercaptoethanol. Cell counts were determined from replicate plates in each experiment.

Ligand and Western blotting. Aliquots of conditioned serum-free medium, conditioned AIB assay buffer, or solubilized cells were brought to 80 μ l with concentrated electrophoresis sample buffer (final concentration, 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue) without 2-mercaptoethanol and heated for 2 min at 100°C. SDS-PAGE was performed according to the method of Laemmli (18) with 12.5% acrylamide resolving gels of 0.75 mm thickness, as described by Hossenlopp et al. (19). Prestained molecular weight standards (Bethesda Research Laboratories, Gaithersburg, MD) included myosin (*M*, 200,000), phosphorylase b (*M*, 97,400), BSA (*M*, 68,000), ovalbumin (*M*, 43,000), carbonic anhydrase (*M*, 29,000), β -lactoglobulin (*M*, 18,400), and lysozyme (*M*, 14,300). Because coupling of the blue chromophore to these standards affects their electrophoretic behavior in SDS-PAGE, the molecular weights of the pre-

stained standards empirically determined by the manufacturer were used to estimate the molecular weights of the IGF binding proteins. Proteins were electrophoretically transferred to nitrocellulose sheets according to the method of Towbin et al. (20). Transfer was accomplished in a GENIE electrophoretic blotter (Idea Scientific Company, Corvallis, OR) using a transfer buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol and applying a current of ~ 0.6 Amp for 1 h.

For ligand blot analysis, the blot was incubated overnight at room temperature in blocking buffer (50 mM Tris-HCl, pH 7.8, with 5% Carnation nonfat dry milk, 2 mM CaCl₂, 0.01% antifoam A emulsion, and 0.05% Triton X-100) with gentle shaking. ¹²⁵I-labeled IGF-I (10⁵ cpm/ml buffer) was then added for 2 h. The blot was washed with fresh blocking buffer and then twice with PBS and dried. Autoradiographs were obtained by exposure of the dried blots to Kodak XAR-5 x-ray film in the presence of a Cronex Lightning Plus intensifying screen at -70°C and were scanned with an LKB Ultrascan XL laser densitometer.

For Western blot analysis, the blot was washed and incubated overnight at room temperature in TBS (50 mM Tris-HCl, pH 7.4, 200 mM NaCl) with 30 mg/ml BSA and 0.02% NaN₃. Rabbit anti-human IGFBP-1 antibody (1:10,000 dilution) in TBS with 10 mg/ml BSA was then added for 1.5 h. The blots were washed three times with TBS containing 0.1% NP-40 and 0.03% Triton X-100, once with TBS, and then incubated for 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5,000 dilution) in TBS with 10 mg/ml BSA. The blots were washed as described above, washed once with AP buffer (100 mM Tris-HCl, pH 9.5, with 100 mM NaCl, and 5 mM MgCl₂), and developed using 66 μl of nitroblue tetrazolium (50 mg/ml in 70% *N,N*-dimethylformamide) and 33 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in 100% *N,N*-dimethylformamide) in 10 ml AP buffer. After 30 min, the blot was washed with PBS with 20 mM EDTA and dried.

IGF binding protein binding studies. IGF-I binding to secreted IGF binding proteins was determined as described by McCusker and Clemmons (21). Briefly, conditioned AIB assay buffer was incubated with ¹²⁵I-labeled IGF-I and increasing amounts of unlabeled IGF-I in 0.1 M Hepes, pH 6.0, with 44 mM NaH₂PO₄, 0.01% Triton X-100, 1 mg/ml BSA, and 0.02% NaN₃ in 1.5 ml microfuge tubes. The final volume was 250 μl. After a 1-h incubation at room temperature, complexes of binding protein and IGF-I were precipitated at 4°C by the addition of 250 μl of 0.4% bovine gamma-globulins in 0.1 M Tris-HCl, pH 7.4, and 500 μl of 25% PEG, followed by centrifugation in an Eppendorf microfuge for 15 min. The supernatant fluid was removed by aspiration, and the lower tip of the tube, containing the pellet, was excised and counted in a Micromedic 4/200 Automatic gamma counter. ¹²⁵I-labeled IGF-I binding was dependent on the amount of conditioned AIB assay buffer in the assay, and experimental results presented in this study were those obtained using amounts of conditioned AIB assay buffer at which the dose-dependent relationship was linear.

Methods of analysis. The data from competition binding studies were analyzed by LIGAND, a computer program developed by Munson and Rodbard (22). The values reported for binding capacities and dissociation constants were obtained from a fit based on one class of sites since the improvement in fit by adding another class of sites was not substantial enough to warrant the additional complexity.

Results

IGF-I stimulation of AIB uptake and thymidine incorporation in normal and CSC fibroblasts. We have reported that fibroblasts from a child with short stature (CSC) were significantly less responsive to stimulation by IGF-I than fibroblasts from normal subjects (6). Fig. 1 shows a representative experiment in which the ED₅₀ of IGF-I-stimulated AIB uptake in this patient's fibroblasts was three times higher than that in normal fibroblasts. In order to minimize potential modulation of IGF-

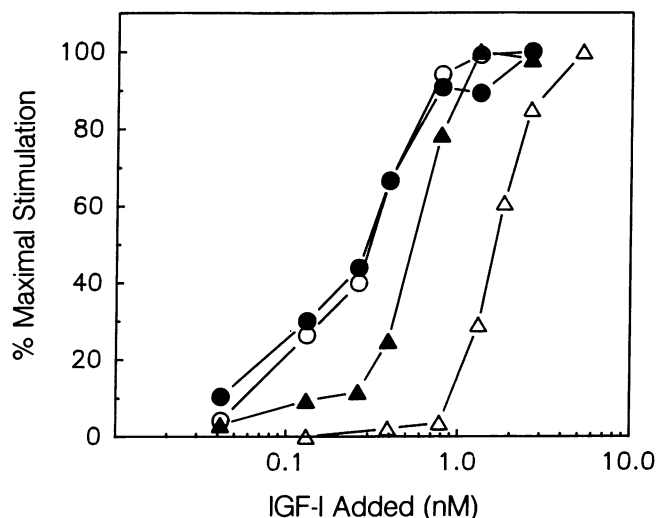


Figure 1. IGF-I and [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I stimulation of AIB uptake in normal and CSC fibroblasts. GM5565 (▲, ●) and CSC (△, ○) fibroblasts were incubated with increasing amounts of IGF-I (▲, △) or [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I (●, ○) for 3 h in a volume of 500 μl. Uptake of 30 μM AIB containing 2 μCi/ml [³H] AIB in 20 min was then measured, as previously described (6). Each point represents the mean of triplicate determinations. The data are presented as the percent of maximal stimulation observed for each dose-response curve. The ED₅₀s for IGF-I stimulation of AIB uptake in GM5565 and CSC fibroblasts were 0.54 nM and 1.63 nM, respectively, and for [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I stimulation were 0.29 nM and 0.31 nM, respectively.

I action by endogenous IGF binding proteins in these assays, we examined fibroblast responsiveness to [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I, an IGF-I variant that has a reduced affinity for human serum IGF binding proteins (9). As shown in Fig. 1, AIB uptake in normal and CSC fibroblasts was comparably stimulated by [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I. Table I summarizes IGF-I and [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I stimulation of AIB uptake in fibroblasts from the patient and from five normal subjects. The ED₅₀s of [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I-stimulated AIB uptake in CSC (0.38 nM) and normal (0.27 nM) fibroblasts were very similar. Furthermore, the mean ED₅₀ of [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I-stimulated AIB uptake was 39.7% and 20.0% of that of IGF-I-stimulated AIB uptake in normal and CSC fibroblasts, respectively. Maximal stimulation of AIB uptake (mean±SE) by IGF-I (2.3±0.1-fold in five normal and 2.1±0.1-fold in CSC fibroblasts) and by [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I (2.4±0.1-fold in five normal and 2.5±0.1-fold in CSC fibroblasts) did not differ significantly in these studies.

Table I also summarizes IGF-I and [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I stimulation of thymidine incorporation into DNA in fibroblasts from the patient and from four normal subjects. The mean ED₅₀ of IGF-I-stimulated thymidine incorporation in the patient's fibroblasts was 2.8 times higher than that in normal fibroblasts. The ED₅₀s of [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I-stimulated thymidine incorporation in CSC (0.09 nM) and in normal (0.12 nM) fibroblasts were very similar. Furthermore, the mean ED₅₀ of [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I-stimulated thymidine incorporation was 7.4% and 2.0% of that of IGF-I-stimulated thymidine incorporation in normal and CSC fibroblasts, respectively. Maximal stimulation of thymidine incorporation

Table I. Biological Activity of $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ in Fibroblasts

	Stimulation of AIB Uptake (ED ₅₀)		Stimulation of Thymidine Incorporation (ED ₅₀)	
	IGF-I	$[Q^3, A^4, Y^{15}, L^{16}]IGF-I$	IGF-I	$[Q^3, A^4, Y^{15}, L^{16}]IGF-I$
	nM		nM	
Normals				
GM5565	0.70±0.08 (3)	0.22±0.01 (3)	1.98±0.29 (6)	0.03±0.01 (6)
GM3349	0.83±0.09 (3)	0.38±0.03 (3)	1.98±0.26 (4)	0.14±0.03 (2)
GM5757	0.94±0.24 (3)	0.19±0.02 (3)	—	—
WUMS1	0.40±0.05 (3)	0.29±0.05 (3)	0.98±0.16 (6)	0.15±0.02 (3)
WUMS2	0.53±0.06 (3)	0.27±0.01 (3)	1.57±0.16 (5)	0.16±0.03 (3)
Mean±SD	0.68±0.22	0.27±0.07	1.63±0.47	0.12±0.06
Patient (CSC)	1.90±0.30 (3)	0.38±0.03 (3)	4.52±0.38 (5)	0.09±0.03 (6)

IGF-I and $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ stimulation of AIB uptake in fibroblasts from the patient and from five normal subjects were measured as described in the legend to Fig. 1, using a 250- μ l assay volume. IGF-I and $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ stimulation of thymidine incorporation in these fibroblasts were measured as described in Methods. The data are expressed as the ED₅₀ of IGF-I or $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ -stimulated AIB uptake² or thymidine incorporation (mean±SE). The number of independently performed experiments is shown in parentheses.

(mean±SE) by IGF-I (2.5±0.3-fold in four normal and 2.5±0.2-fold in CSC fibroblasts) and by $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ (2.6±0.3-fold in four normal and 2.6±0.1-fold in CSC fibroblasts) did not differ significantly in these studies.

Analysis of the IGF-I receptor in normal and CSC fibroblasts. Although we and others (6–8, 23–25) have studied IGF-I binding to fibroblasts after brief treatment with trypsin to remove adherent binding proteins, potential modification of the IGF-I receptor by this treatment may complicate the interpretation of such studies. To compare IGF-I binding to the cell surface of normal and CSC fibroblast monolayers, competition binding studies were performed using IGF-I and $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$. Fig. 2 (left panels) shows Scatchard plots of data from a representative experiment. Scatchard plots of IGF-I and $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ binding to normal and CSC fibroblast monolayers were linear. In the experiment shown in Fig. 2 A, normal fibroblasts bound 13.4 fmol of IGF-I per well and CSC fibroblasts bound 58.8 fmol of IGF-I per well. In five experiments, the IGF-I binding capacity (mean±SE) of CSC fibroblasts (146,600±23,000 sites/cell) was 2.9 times higher than that of normal (GM5565) fibroblasts (49,900±10,000 sites/cell). In contrast, as shown in Fig. 2 B, $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ binding to normal and CSC fibroblast monolayers was similar. We have compared $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ binding to fibroblasts from the patient and from five normal subjects. In four experiments, the $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ binding capacity (mean±SD) of CSC fibroblasts (13,600±4,400 sites/cell) did not differ from that of five normal fibroblasts (13,900±2,500 sites/cell). The dissociation constants of $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ binding to CSC and normal fibroblasts were 0.39 nM and 0.36–0.50 nM, respectively, indicating that the receptors have a similar affinity for $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$.

α IR-3, the MAb to the IGF-I receptor, has been shown to block IGF-I binding to human placental membranes, to human circulating monocytes, and to suspended human fibroblasts (26, 27). Inhibition of IGF-I and $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ binding to fibroblast monolayers by α IR-3 is shown in Fig. 2 (right panels). Initial experiments indicated that 2.3 μ g/ml α IR-3 maximally inhibited ¹²⁵I-labeled IGF-I and ¹²⁵I-labeled $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ binding to normal fibroblast monolayers.

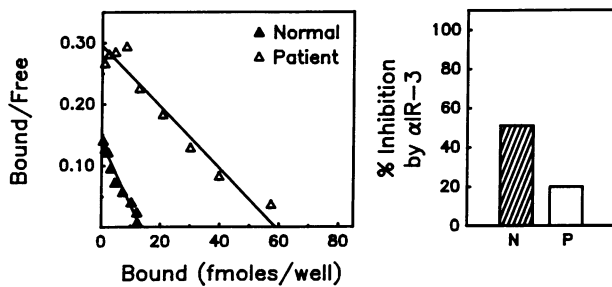
¹²⁵I-labeled IGF-I binding to normal and CSC fibroblasts was inhibited 55% and 19%, respectively, by 2.3 μ g/ml α IR-3, as shown in Fig. 2 A. In contrast, ¹²⁵I-labeled $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ binding to normal and CSC fibroblasts was inhibited 93% and 88%, respectively, by α IR-3, as shown in Fig. 2 B. Similar results were obtained in four other normal cell lines (data not shown).

The receptor to which IGF-I binds in these fibroblasts was determined by affinity cross-linking studies, as shown in Fig. 3. ¹²⁵I-labeled IGF-I was cross-linked predominantly to proteins that migrated under nonreducing conditions as complexes with apparent M_r > 300,000. These proteins correspond to the $(\alpha\beta)_2$, the $(\alpha\beta)(\alpha\beta)$, and the $(\alpha\beta)_2$ forms of the IGF-I receptor (28). After reduction with 2-mercaptoethanol, these complexes migrated with an apparent M_r 135,000 (see Fig. 7 B). Cross-linking of ¹²⁵I-labeled IGF-I was markedly inhibited by unlabeled IGF-I, was inhibited by unlabeled IGF-II, and was not inhibited by unlabeled insulin. Normal and CSC fibroblasts exhibited no qualitative or quantitative differences in IGF-I receptor cross-linking. Taken together with the normal responsiveness of CSC fibroblasts to $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$, these studies indicate that the resistance to IGF-I action in the patient's fibroblasts is not caused by a defective IGF-I receptor.

Analysis of IGF binding proteins in normal and CSC fibroblasts. Since $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ interacts minimally with IGF binding proteins, the comparable stimulation of AIB uptake and thymidine incorporation by and binding of $[Q^3, A^4, Y^{15}, L^{16}]IGF-I$ in normal and CSC fibroblasts suggested that IGF-I sensitivity in the patient's fibroblasts was reduced by IGF binding proteins. To examine IGF binding proteins in normal and CSC fibroblasts directly, fibroblast binding proteins were collected and analyzed as described in Methods. Fig. 4 shows the ligand blot of binding proteins secreted into serum-

2. We have noted a small, but reproducible, increase in ED₅₀s of IGF-I-stimulated AIB uptake when a volume of 250 μ l is used in the AIB uptake assay. Compare ED₅₀s in this table to those obtained in fibroblasts from 11 normal subjects (0.42±0.12 nM), from 127 short children (0.35±0.11 nM), and from the patient (1.40±0.24 nM), using a 500- μ l assay volume.

A IGF-I



B [Gln³,Ala⁴,Tyr¹⁵,Leu¹⁶]IGF-I

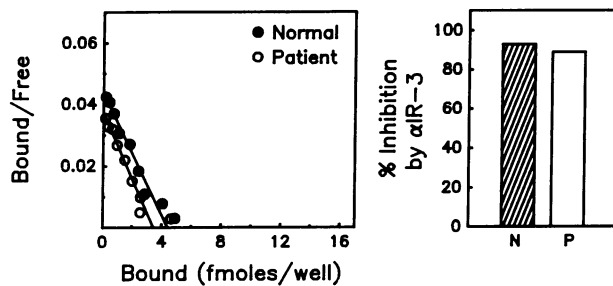


Figure 2. IGF-I and [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I binding to normal and CSC fibroblast monolayers. (A and B, left) Scatchard plots of IGF-I and [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I binding. Competition binding studies were performed at 4°C as described in Methods, using GM5565 (1.9×10^5 cells/well) or CSC (1.8×10^5 cells/well) fibroblast monolayers. Binding assays contained 7,300 cpm (0.02 nM) of ¹²⁵I-labeled IGF-I (A) or 7,900 cpm (0.02 nM) of ¹²⁵I-labeled [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I (B) and 0–6.54 nM of unlabeled IGF-I (A) or [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I (B) in a final volume of 250 μ l. Each point represents the mean of duplicate determinations. Binding data were analyzed by LIGAND (22). *N*, the ratio of nonspecifically bound ligand to free ligand, was 0.009 and 0.010 in A and 0.004 and 0.006 in B for GM5565 and CSC fibroblasts, respectively. Nonspecific binding, *N* \times *F*, has been subtracted. The solid lines are computer-generated fits for a one-site binding model. In this experiment, the dissociation constants for IGF-I and [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I binding to GM5565 fibroblasts were 0.39 nM and 0.40 nM and to CSC fibroblasts were 0.80 nM and 0.37 nM, respectively. (A and B, right) Inhibition of IGF-I and [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I binding by α IR-3. Binding of ¹²⁵I-labeled IGF-I (A) or ¹²⁵I-labeled [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I (B) to GM5565 (cross-hatched bars) and CSC (open bars) fibroblast monolayers was measured in the absence and presence of 2.3 μ g/ml α IR-3. In the absence of α IR-3, 11.6% and 17.0% of ¹²⁵I-labeled IGF-I and 4.7% and 4.8% of ¹²⁵I-labeled [Q³,A⁴,Y¹⁵,L¹⁶]IGF-I was specifically bound to GM5565 and CSC fibroblasts, respectively. Data are presented as the percent of total binding inhibited by α IR-3 (mean of two independently performed experiments).

free medium over 2 d. The most prominent proteins in conditioned serum-free medium from both normal and CSC fibroblasts migrated on SDS-PAGE with apparent *M_r* 46,400 and 42,900. These appear to be identical in size to the major doublet of IGF binding proteins in human serum. In serum, a growth hormone-dependent *M_r* 150,000 complex (29) that dissociates upon acidification to yield free IGFs and an acid-stable subunit, termed IGFBP-3, contains most of the circulating IGFs. Martin and Baxter (30) showed, and we have confirmed

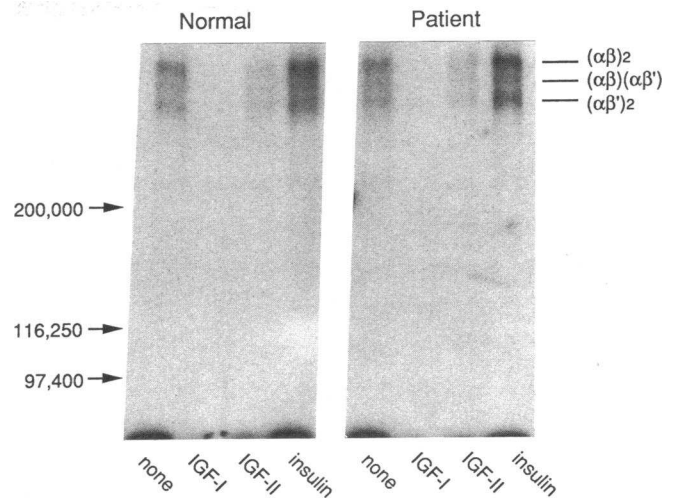


Figure 3. Affinity cross-linking analysis of the IGF-I receptor in normal and CSC fibroblasts. Day 6 GM5565 and CSC fibroblasts were incubated for 4 h at 15°C with 0.2 nM ¹²⁵I-labeled IGF-I in the absence or presence of 100 ng/ml of unlabeled IGF-I, unlabeled IGF-II, or unlabeled insulin, as indicated. Affinity cross-linking with 0.1 mM disuccinimidyl suberate was performed as previously described (17). The autoradiograph of the radioligand/receptor complexes, analyzed by SDS-PAGE (5% acrylamide resolving gel) under nonreducing conditions, is shown. The migration of molecular weight standards is indicated.

(data not shown), that the doublet in fibroblast-conditioned serum-free medium reacts with an antibody raised against human IGFBP-3. Two other proteins with apparent *M_r* 32,000 and 26,800 were also present in conditioned serum-free medium from both normal and CSC fibroblasts.³ All of these IGF binding proteins appeared to be secreted in increased amounts by CSC fibroblasts. To examine the possibility that the serum-free medium was contaminated by binding proteins from FCS in which the cells had been grown, we analyzed DME containing 10% FCS. The electrophoretic profile of IGF binding proteins in FCS was markedly different than that in conditioned serum-free medium. The major protein in FCS (apparent *M_r* 36,800) did not appear to be present in the conditioned serum-free medium from fibroblasts.

To examine binding protein production during the AIB uptake assay, incubation conditions were scaled-up as described in Methods. IGF binding proteins secreted by normal and CSC fibroblasts were first compared in competition bind-

3. The electrophoretic profiles of IGF binding proteins secreted by fibroblasts into serum-free medium are very similar to those published recently by Conover et al. (31) and Hill et al. (32), although the estimated molecular weights of the binding proteins differ. It is likely that the binding proteins with apparent *M_r* 46,400, 42,900, 32,000, and 26,800 in our study correspond to those with apparent *M_r* 41,500–42,000, 37,000–39,000, 28,000–30,000, and 23,000–25,000, respectively, in their studies. The differences reported in apparent molecular weights may result from the conditions of SDS-PAGE (a 12.5% acrylamide resolving gel used in our study and a 5–15% gradient acrylamide resolving gel employed in the study of Conover et al. [31]). In addition, we have used the plot of mobility vs. the logarithm of the molecular weights of the pre-stained standards empirically determined by the manufacturer rather than the logarithm of the reported molecular weights to estimate the molecular weights of the IGF binding proteins.

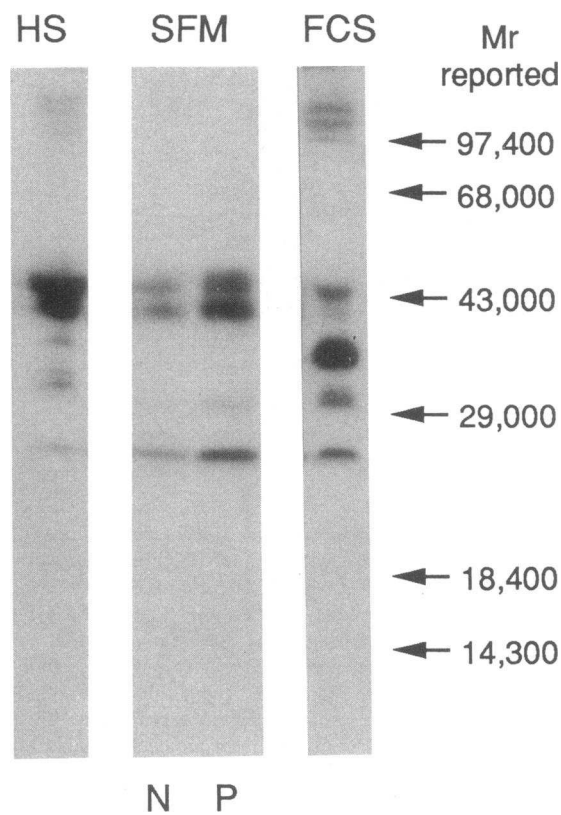


Figure 4. Ligand blot analysis of IGF binding proteins secreted by normal and CSC fibroblasts. GM5565 (*N*) and CSC (*P*) fibroblasts were treated for 2 d with serum-free medium. The conditioned serum-free medium (*SFM*) was collected, and 20- μ l aliquots were analyzed by ligand blotting, as described in Methods. A 1.6- μ l aliquot of human serum (*HS*) and a 20- μ l aliquot of DME containing 10% FCS were also analyzed. The migration of prestained molecular weight standards is indicated.

ing studies. Fig. 5 shows Scatchard plots of data from a representative experiment. Scatchard plots of IGF-I binding to 3-h conditioned AIB assay buffer from normal and CSC fibroblasts were linear. In this experiment, the binding capacities of conditioned AIB assay buffer from normal and CSC fibroblasts were 0.24 pmol of IGF-I bound/ml and 0.49 pmol of IGF-I bound/ml, respectively. Analysis of IGF-I binding to conditioned AIB assay buffer from three separate collections indicated that the IGF-I binding capacity of conditioned AIB assay buffer from CSC fibroblasts was 2.0–2.7 times higher than that from normal fibroblasts.⁴ The dissociation constants (mean \pm SD) of

4. The difference in binding capacity of conditioned AIB assay buffer from normal (GM5565) and CSC fibroblasts obtained from Scatchard plots, as shown in Fig. 5, was comparable to that obtained from binding of ¹²⁵I-labeled IGF-I only (2.4 times). In a separate experiment, we compared binding of ¹²⁵I-labeled IGF-I only to conditioned AIB assay buffer from fibroblasts from the patient and from five normal subjects (including GM5565). Conditioned AIB assay buffer from CSC fibroblasts bound 1.3–2.9 times more ¹²⁵I-labeled IGF-I. When corrected for cell counts, conditioned AIB assay buffer from CSC fibroblasts bound 1.4–2.6 times more ¹²⁵I-labeled IGF-I than that from four of five normal fibroblasts. Conditioned AIB assay buffer from WUMS2 fibroblasts bound as much ¹²⁵I-labeled IGF-I as CSC fibroblasts when corrected for cell counts, despite its similarity to fibroblasts from the normal subjects in other assays (see Table I).

IGF-I binding to conditioned AIB assay buffer from normal and CSC fibroblasts were 0.056 \pm 0.006 nM and 0.067 \pm 0.006 nM, respectively, indicating that the composite affinity of binding proteins secreted by normal and CSC fibroblasts for IGF-I is similar.

IGF binding proteins that were secreted into AIB assay buffer during a 3-h collection and that were cell-associated at the end of the collection were then analyzed by ligand blotting. As shown in Fig. 6, the electrophoretic profile of IGF binding proteins secreted into AIB assay buffer in 3 h was similar to that secreted into serum-free medium over 2 d (see Fig. 4). We estimated the amounts of IGF binding proteins by densitometric scanning of autoradiographs from six independently performed ligand blots using conditioned AIB assay buffer and solubilized cells from three separate collections. Analysis of equivalent aliquots of conditioned AIB assay buffer and solubilized cells indicated that most of the binding protein present after a 3-h collection was secreted into AIB assay buffer. Overall, by densitometric scanning of autoradiographs, we estimated that 1.4–1.8 times more binding protein was secreted by CSC fibroblasts than by normal (GM5565) fibroblasts. Specifically, CSC fibroblasts secreted 1.3 \pm 0.16, 1.8 \pm 0.34, and 2.2 \pm 0.54 (mean \pm SD) times more of the *M*_r 46,400/42,900 doublet, the *M*_r 32,000 binding protein, and the *M*_r 26,800 binding protein, respectively, than normal fibroblasts.

Interestingly, as shown in Fig. 6, the electrophoretic profile

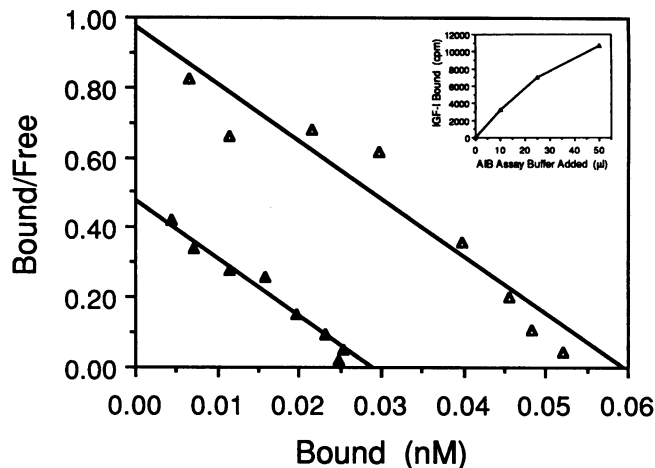


Figure 5. Scatchard plots of IGF-I binding to conditioned AIB assay buffer from normal and CSC fibroblasts. AIB assay buffer was collected from untreated GM5565 (\blacktriangle) and CSC (\triangle) fibroblasts after a 3-h incubation at 37°C. Cell counts were 3.24×10^6 cells/plate and 4.2×10^6 cells/plate for GM5565 and CSC fibroblasts, respectively, in this experiment. IGF-I binding studies were performed as described in Methods, using 30 μ l of conditioned AIB assay buffer. The inset shows that the relationship of ¹²⁵I-labeled IGF-I bound to the amount of conditioned AIB assay buffer (from CSC fibroblasts) in the assay is linear at 30 μ l. Binding assays contained 11,900 or 23,700 cpm (0.015 or 0.030 nM) of ¹²⁵I-labeled IGF-I and 0–65.4 nM of unlabeled IGF-I in a final volume of 250 μ l. Each point represents the mean of duplicate determinations. Binding data were analyzed by LIGAND (22). *N* was 0.0841 and 0.0978 for GM5565 and CSC fibroblasts, respectively, and nonspecific binding has been subtracted. The solid lines are computer-generated best fits for a one-site binding model. The dissociation constants for IGF-I binding to conditioned AIB assay buffer from GM5565 and CSC fibroblasts in this experiment were 0.060 nM and 0.061 nM, respectively.

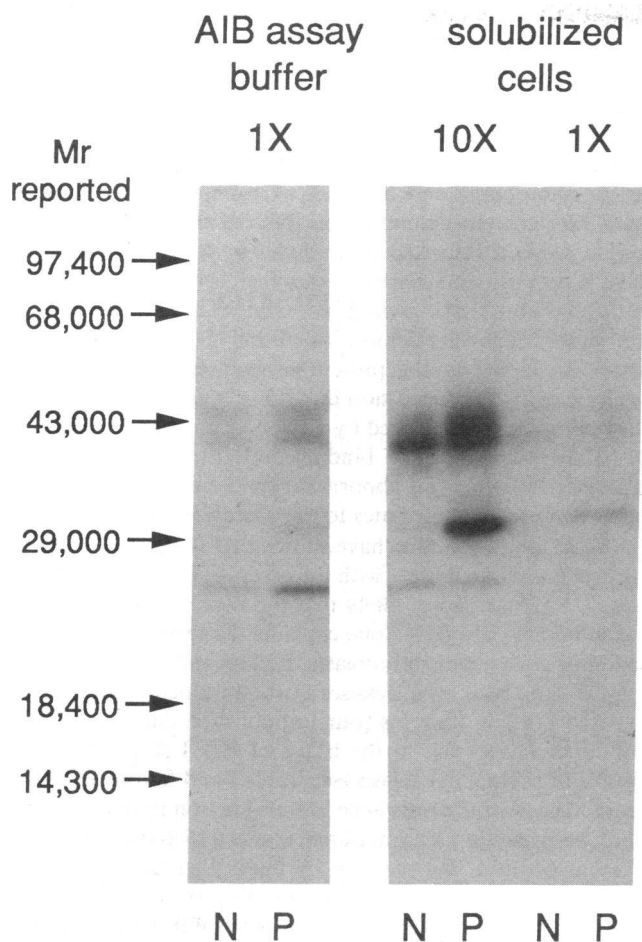


Figure 6. Ligand blot of secreted and cell-associated IGF binding proteins in normal and CSC fibroblasts. AIB assay buffer (6.55 ml) was collected from untreated GM5565 (N) and CSC (P) fibroblasts after a 3-h incubation at 37°C. The fibroblasts were then washed and solubilized in electrophoresis sample buffer (500 μ l). Equivalent aliquots of conditioned AIB assay buffer (60 μ l) and of solubilized cells (4.5 μ l) and a 10 \times aliquot of solubilized cells (45 μ l) were analyzed by ligand blotting, as described in Methods. In this autoradiograph, the densitometric signal of the M_r 32,000 binding protein in the 10 \times aliquot of solubilized CSC cells was 10.9 times higher than that in the 1 \times aliquot, confirming that the autoradiographic exposure gave densitometric signals in the linear range. The migration of prestained molecular weight standards is indicated.

of binding proteins that were cell-associated in CSC fibroblasts differed markedly from that of secreted binding proteins. Overall, by densitometric scanning of autoradiographs, we estimated that 1.8–2.9 times more binding protein was cell-associated in CSC fibroblasts than in normal (GM5565) fibroblasts. The major difference between normal and CSC fibroblasts was the striking amount of M_r 32,000 binding protein that was cell-associated in CSC fibroblasts. 1.4 ± 0.28 , 10.0 ± 1.41 , and 2.4 ± 0.57 (mean \pm SD) times more of the M_r 46,400/42,900 doublet, the M_r 32,000 binding protein, and the M_r 26,800 binding protein, respectively, were cell-associated in CSC fibroblasts than in normal fibroblasts.

To demonstrate that the M_r 32,000 cell-associated IGF binding protein in solubilized CSC fibroblasts is on the cell surface, affinity cross-linking studies were performed, as shown in Fig. 7. 12.5% or 3–12.5% gradient acrylamide resolving gels

were used in order to detect radioligand/binding protein complexes. In normal fibroblasts, 125 I-labeled IGF-I was cross-linked predominantly to proteins that migrated under nonreducing conditions (Fig. 7 A) as complexes with apparent M_r 53,400/48,900. These molecular weights are appropriate for complexes of cross-linked IGF-I (M_r 7,649) with the M_r 46,400/42,900 doublet. In CSC fibroblasts, 125 I-labeled IGF-I was cross-linked to an additional protein which migrated under nonreducing conditions (Fig. 7 A) as a complex with apparent M_r 41,800. This molecular weight is appropriate for a complex of cross-linked IGF-I with the M_r 32,000 binding protein.

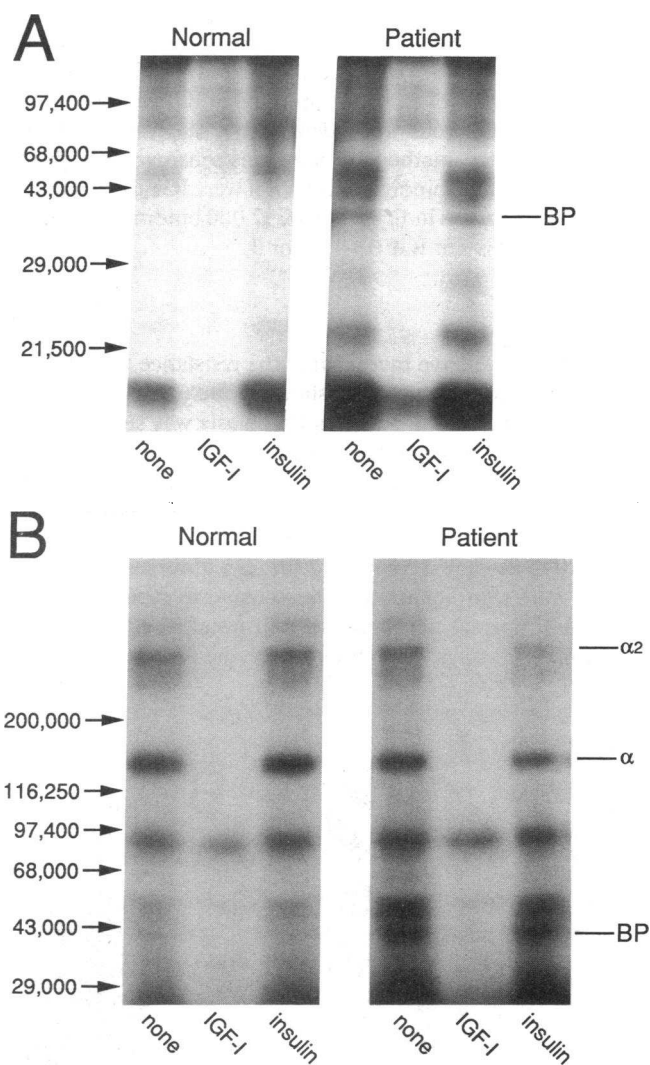


Figure 7. Affinity cross-linking analysis of cell-associated IGF binding proteins in normal and CSC fibroblasts. Day 8 GM5565 (4.64×10^6 cells/plate) and CSC (4.49×10^6 cells/plate) fibroblasts were incubated at 4 h at 15°C with 0.2 nM 125 I-labeled IGF-I in the absence or presence of 125 ng/ml of unlabeled IGF-I or unlabeled insulin, as indicated. Affinity cross-linking with 0.1 mM disuccinimidyl suberate was performed as previously described (17) except that EDTA was omitted from the quenching buffer. (A) The autoradiograph of the radioligand/binding protein complexes, analyzed by SDS-PAGE (12.5% acrylamide resolving gel) under nonreducing conditions. (B) The autoradiograph of the radioligand/binding protein and receptor complexes, analyzed by SDS-PAGE (3–12.5% gradient acrylamide resolving gel) under reducing conditions. The migration of molecular weight standards is indicated.

Under reducing conditions (Fig. 7 B), ^{125}I -labeled IGF-I was cross-linked to this binding protein and to the α subunit (and the covalently cross-linked α_2 dimer) of the IGF-I receptor. Cross-linking of ^{125}I -labeled IGF-I was markedly inhibited by unlabeled IGF-I but not by unlabeled insulin.

Identification of the M_r 32,000 IGF binding protein. We have performed studies to compare the characteristics of the M_r 32,000 binding protein in CSC fibroblasts with the three distinct, well-characterized classes of IGF binding proteins (3). Ligand blot analysis demonstrated that the M_r 32,000 binding protein is similar in size to IGFBP-1 and different from IGFBP-2 (Fig. 8 A) and IGFBP-3 (see Fig. 4). It is also different from the major binding protein in human CSF, which is similar in size to IGFBP-2. Nevertheless, the M_r 32,000 binding protein did not cross-react with an antibody against human IGFBP-1 (Fig. 8 B). Even small amounts of IGFBP-1 secreted by human HepG2 cells were identified in the Western blot, suggesting that the method we used was sensitive enough to detect the M_r 32,000 binding protein if it were IGFBP-1. Taken together, these results indicate the M_r 32,000 binding protein in CSC fibroblasts is not IGFBP-1, 2, or 3.

Discussion

In this report, we have investigated the resistance to IGF-I in fibroblasts from one child with short stature (6). The cause of IGF-I resistance in this patient's fibroblasts was suggested by experiments with an IGF-I variant, $[\text{Q}^3, \text{A}^4, \text{Y}^{15}, \text{L}^{16}]$ IGF-I, which has a 600-fold reduced affinity for IGF binding proteins (9). Binding of this IGF-I variant to normal fibroblasts and to the patient's fibroblasts was similar and was inhibited by $\alpha\text{IR}-3$, a MAb to the IGF-I receptor. Although fibroblasts from the patient were significantly less responsive to stimulation by IGF-I than normal fibroblasts in AIB uptake and thymidine incorporation assays, their response to the IGF-I variant was

normal. Taken together with affinity cross-linking studies, these results indicated that the IGF-I receptor in this patient's fibroblasts was structurally and functionally intact and suggested that IGF-I sensitivity was reduced by increased amounts of endogenous IGF binding proteins. To confirm this directly, secreted and cell-associated IGF-I binding proteins from this patient's fibroblasts were analyzed. IGF-I binding studies and ligand blot analysis demonstrated that the amount of binding protein secreted into AIB assay buffer by the patient's fibroblasts was approximately two times higher than that secreted by normal fibroblasts. Ligand blot analysis and affinity cross-linking studies further demonstrated that the amount of a cell surface M_r 32,000 binding protein was increased 10-fold. These results support the conclusion that IGF-I sensitivity in this patient's fibroblasts is reduced by an abnormal production and/or cell association of IGF binding proteins. It remains to be established whether this abnormality is present in other IGF-I target tissues and contributes to this child's short stature.

While binding studies have shown that secreted IGF binding proteins may interfere with the access of IGFs to cell receptors (7, 8, 25), it is not likely that the twofold increase in secreted binding proteins alone explains the resistance to IGF-I action in our patient's fibroblasts. Reducing the assay volume from 500 to 250 μl , which causes a twofold concentration of the secreted binding proteins (our unpublished data), results in only a small increase in the ED_{50} s of IGF-I stimulated AIB uptake in normal fibroblasts (see Table I and footnote 2). It is more likely that the resistance to IGF-I action in our patient's fibroblasts results from interference with IGF-I-dependent activation of the IGF-I receptor by the cell surface M_r 32,000 binding protein. In competition binding studies, fibroblasts from the patient bound 2.9 times more IGF-I than normal (GM5565) fibroblasts but similar amounts of the IGF-I variant. In addition, a MAb to the IGF-I receptor blocked IGF-I binding to the patient's fibroblasts less potently than to normal

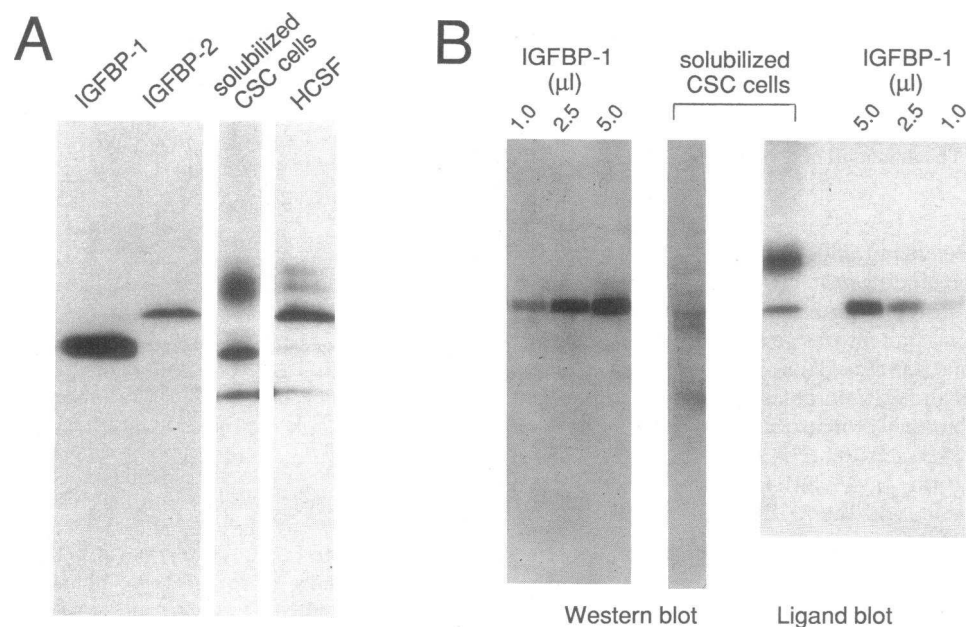


Figure 8. Ligand and Western blot comparison of the CSC fibroblast M_r 32,000 binding protein and IGFBP-1 and IGFBP-2. CSC fibroblasts were washed and solubilized in 500 μl electrophoresis sample buffer. HepG2 and Jurkat cells were grown for ~ 64 h in serum free-media that was collected and clarified by low speed centrifugation. (A) Ligand blot of a 10- μl aliquot of conditioned medium from HepG2 cells, a 60- μl aliquot of conditioned medium from Jurkat cells, a 15- μl aliquot of 10 \times -concentrated human CSF (HCSF), and a 45- μl aliquot of solubilized CSC cells. (B) Western and ligand blot of solubilized CSC cells and IGFBP-1. Aliquots (1–5 μl , as indicated) of conditioned medium from HepG2 cells and a 90- μl aliquot of solubilized CSC cells were analyzed by ligand blotting (right half of filter) and Western blotting (left half of filter), as described in

Methods. Complexes of IGFBP-1 and rabbit anti-human IGFBP-1 antibody were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG.

fibroblasts. Since binding studies were performed at 4°C, a temperature at which no binding protein is secreted into the medium (our unpublished data), secreted binding proteins did not interfere with IGF-I binding to cell receptors in these studies. Ligand blot analysis confirmed that 1.8–2.9 times more binding protein was associated with the patient's fibroblasts than with normal (GM5565) fibroblasts and demonstrated that this increase resulted largely from a 10-fold increase in the cell association of the *M*, 32,000 binding protein. The mechanism by which the cell surface *M*, 32,000 binding protein interferes with IGF-I action in the patient's fibroblasts remains to be determined.

The identity of the fibroblast *M*, 32,000 binding protein is not clear. Both IGFBP-1 (13, 33, 34) and IGFBP-2 (14, 35) contain an RGD sequence near their carboxyl termini which may interact with specific cell-surface receptors in the integrin superfamily (36). Hill et al. (32) reported that fibroblasts secrete a binding protein with apparent *M*, 30,000 that reacts with an antibody raised against IGFBP-1 purified from human amniotic fluid. In contrast, Martin and Baxter (30) reported that no binding protein was detectable in fibroblast-conditioned medium by RIA or by immunoprecipitation of affinity cross-linked complexes using another antibody raised against human amniotic fluid IGFBP-1. Their results are supported by the recent observation by Conover et al. (31) that RNA isolated from fetal or postnatal human fibroblasts did not hybridize on Northern blot analysis with an IGFBP-1 cDNA probe from the HepG2 cell line. Since the nucleotide sequences of IGFBP-1 cDNAs from a human decidua library (33) and a human placenta library (34) and from a human HepG2 library (13) are nearly identical, these apparently conflicting results are not readily explained. It is possible that differences existed in the fibroblast lines used or in the specificities of the antibodies available in these studies. Alternatively, it is possible that the secretion of IGFBP-1 is developmentally regulated, since Hill et al. (32) reported that the ability of fibroblasts to secrete IGFBP-1 was related to cell density, being greatest in sparse cultures. The expression of IGFBP-2 in human fibroblasts has not been reported, but the mRNA expression of this binding protein in the rat has been shown to be highest in fetal tissues and greatly reduced in the corresponding adult tissues (35). Martin and Baxter (30) reported that a (¹²⁵I-labeled IGF-II cross-linked) human fibroblast binding protein similar in size to IGFBP-1 could be immunoprecipitated using an antibody against IGFBP-3. This antibody (and an antibody against IGFBP-2 [37]) have also been reported to cross-react with a binding protein in conditioned medium from the SV40-transformed human fibroblast line AG2804 (38). We compared the characteristics of the cell-associated *M*, 32,000 binding protein in CSC fibroblasts with these well-characterized IGF binding proteins. The *M*, 32,000 binding protein is similar in size to IGFBP-1, but it does not cross-react with an antibody against purified human amniotic fluid IGFBP-1. It is smaller than IGFBP-2. Finally, it does not appear to be analogous to the SV40-transformed human fibroblast binding protein since we have found that it is not detected by Western blot analysis or by immunoprecipitation of affinity cross-linked complexes using an antibody against IGFBP-3 (our unpublished data). Martin et al. (37) recently reported that the secretion of a human fibroblast binding protein doublet with apparent *M*, 29,000/31,000 could be greatly enhanced by IGF-I and IGF-II; by Western blot analysis, this binding protein did not cross-react with anti-

bodies against IGFBP-1, 2, and 3. Whether this secreted binding protein is analogous to the cell-associated *M*, 32,000 binding protein in CSC fibroblasts remains to be determined.

The physiological relevance of cell surface binding proteins has not been clearly defined. DeMellow and Baxter (39) reported that coincubation of IGF-I with purified IGFBP-3 inhibited IGF-I-stimulated thymidine incorporation in human neonatal fibroblasts but that preincubation of fibroblasts with IGFBP-3 before adding IGF-I resulted in a potentiation of IGF-I action. The adherence of IGFBP-3 to the cell surface was not examined in these studies, although our study (see Fig. 7) and that of Clemmons et al. (8) suggest that a small amount of IGFBP-3 may adhere to the cell surface. Recent studies in human choriocarcinoma JEG-3 cells demonstrated that purified IGFBP-1 inhibited the maximal stimulation of AIB uptake by 10 ng/ml IGF-I (40), but ¹²⁵I-labeled IGFBP-1 alone or complexed to IGF-I did not bind to the JEG-3 cell surface in these studies. In contrast, Elgin et al. (41) reported that purified IGFBP-1 potentiated IGF-I stimulated DNA synthesis in human, mouse embryo, and chick embryo fibroblasts and porcine aortic smooth muscle cells. Subsequent studies revealed two forms of this binding protein with different biologic actions (42). One form, designated peak B, enhanced IGF-I stimulation of [³H]thymidine incorporation into smooth muscle cell DNA, and a second form, peak C, prevented this response to peak B and IGF-I. Peak B was shown to adhere to cell surfaces whereas peak C did not. Further elucidation of IGF binding protein production and the regulation thereof in CSC fibroblasts may provide important insights into the physiological role of cell surface binding proteins in IGF action.

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