Four structural analogs of human insulin-like growth factor I (hIGF-I) have been prepared by site-directed mutagenesis of a synthetic IGF-I gene and subsequent expression and purification of the mutant protein from the conditioned media of transformed yeast. [Phe\(^1\), Val\(^5\), Asn\(^{16}\), Gln\(^{24}\), His\(^{30}\), Ser\(^{32}\), His\(^{33}\), Glu\(^{37}\), Tyr\(^{38}\), Leu\(^{42}\)]IGF-I (B-chain mutant), in which the first 16 amino acids of hIGF-I were replaced with the first 17 amino acids of the B-chain of insulin, has >1,000-, 100-, and 2-fold reduced potency for human serum binding proteins, the rat liver type 2 IGF receptor, and the human placental type 1 IGF receptor, respectively. The B-chain mutant also has 4-fold increased affinity for the human placental insulin receptor. [Gln\(^{7}\), Ala\(^{8}\)]IGF-I has 4-fold reduced affinity for human serum binding proteins, but is equipotent to hIGF-I at the types 1 and 2 IGF and insulin receptors. [Tyr\(^{15}\), Leu\(^{16}\)]IGF-I has 4-fold reduced affinity for human serum binding proteins and 10-fold increased affinity for the insulin receptor. This peptide is also equipotent to hIGF-I at the types 1 and 2 IGF receptors. The peptide in which these four-point mutations are combined, [Gln\(^{7}\), Ala\(^{8}\), Tyr\(^{15}\), Leu\(^{16}\)]IGF-I, has 600-fold reduced affinity for the serum binding proteins. This peptide has 10-fold increased potency for the insulin receptor, but is equipotent to hIGF-I at the types 1 and 2 IGF receptors. All four of these mutants stimulate DNA synthesis in the rat vascular smooth muscle cell line A10 with potencies reflecting their potency at the type 1 IGF receptor. These studies identify some of the domains of hIGF-I which are responsible for maintaining high-affinity binding with the serum binding protein and the type 2 IGF receptor. In addition, these peptides will be useful in defining the role of the type 2 IGF receptor and serum binding proteins in the physiological actions of hIGF-I.

Human insulin-like growth factor I (hIGF-I)\(^1\) is a 70-amino-

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The abbreviations used are: hIGF-I, human insulin-like growth factor I; IGF, insulin-like growth factor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography.

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Structural Analogs of Human Insulin-like Growth Factor I with Reduced Affinity for Serum Binding Proteins and the Type 2 Insulin-like Growth Factor Receptor*

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IGF-I Analogues with Reduced Affinity for Serum Binding Proteins

Expression and Purification of Mutant hIGF-I Peptides

Saccharomyces cerevisiae strain BJ1905 (MATa, leu2, trpl, ural, pohl-1122, pep4-3, cir10) was a gift from Dr. Elizabeth Jones, Carnegie Mellon University. Yeast were transformed as described previously (20), and transformants were selected on leucine minus plates. Cells were grown to saturation in 1 lit of 5 x Leu- media, pH 4.8, containing 0.85% yeast nitrogen base without amino acids and ammonium sulfate supplements, 4% glucose, 0.6% sodium hydroxide, 0.03% l-lysine, 0.02% l-tryptophan, 0.02% uracil, 0.02% adenine, 0.01% l-arginine, 0.005% methionine, 0.005% l-histidine, 29 µM ferric chloride, 25 µM zinc sulfate, and 1% succinic acid. Cells were removed by centrifugation at 3,000 x g. The cleared supernatant was mixed with 10 µg of Bio-Rex 70 equilibrated in 0.1 M ammonium acetate, pH 7.5, 50 mM MgSO4, and 0.1% bovine serum albumin for 1 h at 20 °C. Samples were filtered over Whatman GF/F filters presoaked in 0.1% polyethyleneimine to separate bound to free ligand. The incubation tubes and filters were washed four times with 2.5 ml of cold assay buffer (minus bovine serum albumin). Less than 5% of the ligand bound to filters in the absence of membranes. Placental membranes bound 38% of the ligand in the absence of competing peptides. Nonspecific binding of the ligand to placental membranes was measured by adding excess amounts of unlabeled [Thr3]hIGF-I (0.3 µM) to the incubation, and this routinely represented 5% of the total binding of ligand to membranes.

Rat Liver Membrane—[Thr3]hIGF-I was used for iodosination in all studies. [125I]hIGF-I (50–80 Ci/mg, 50 fmol) and human placental membranes (0.2 mg) were incubated in the presence or absence of competing peptides in 0.2 ml of 0.1 M Hepes, pH 7, containing 120 mM NaCl, 5 mM KCl, 0.12 mM MgSO4, and 0.05% bovine serum albumin for 1 h at 20 °C. Samples were filtered over EHWP filters, and the incubation tubes and filters were washed four times with 2.5 ml of cold assay buffer (minus bovine serum albumin). Less than 5% of the ligand bound to the filters in the absence of membranes. Placental membranes bound 38% of the ligand in the absence of competing peptides. Nonspecific binding of the ligand to placental membranes was measured by adding excess amounts of unlabeled [Thr3]hIGF-I (0.3 µM) to the incubation, and this routinely represented 5% of the total binding of ligand to membranes.

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presence of crude binding protein and in the absence of competing peptides. An excess of [Thr⁵]IGF-I (0.1 μM) inhibited greater than 99% of the binding of ligand to the crude binding protein.

For cross-linking studies, ligand and acid-treated human serum were incubated with or without competing peptides as above for 22 h at 4 °C. Diisuccinimidyl suberate (final concentration 1.5 mM) was added at 22 °C. After 12 min, trichloroacetic acid was added to a final concentration of 10%, and proteins were precipitated on ice for 15 min. The pellet was recovered by centrifugation and washed with 10% trichloroacetic acid. The pellets were solubilized in sodium dodecyl sulfate gel buffer, neutralized, and run on 12% acrylamide gels. The dried gels were exposed to film for 3 days.

Cell-based Assays—The measurement of [methyl-¹²⁵I]thymidine incorporation into DNA in A10 cells has been described in detail elsewhere (14).

Fig. 2. Sequences of the hIGF-I mutant genes and the protein analogs, A, hIGF-I; B, B-chain mutant; C, [Tyr¹⁵,Leu¹⁶]IGF-I; D, [Gln³,Ala⁴]IGF-I; and E, [Gln³,Ala⁴,Tyr¹⁵]IGF-I.

Fig. 3. Bio-Gel P-10 purification of B-chain mutant. The receptor-active fractions from the Bio-Rex 70 purification of 1 liter of conditioned media were concentrated and loaded onto a Bio-Gel P-10 column and eluted as described under “Experimental Procedures.” An aliquot of each fraction was assayed for receptor activity. Each fraction was 12 ml, and data are presented as the micrograms of receptor-active material per fraction.

Fig. 4. Reverse-phase HPLC purification of B-chain mutant (A), [Tyr¹⁵,Leu¹⁶]IGF-I (B), [Gln³,Ala⁴]IGF-I (C), and [Gln³,Ala⁴,Tyr¹⁵]IGF-I (D). Active fractions from Bio-Gel P-10 chromatography were applied to a C₁₈ Bondapak column and eluted with a 15-50% acetonitrile gradient in 0.05% trifluoroacetic acid. Aliquots of each fraction were assayed for their ability to inhibit the binding of ¹²⁵I-IGF-I to human placental membranes. The receptor-active peptide loaded was 193 μg for A, 396 μg for B, 232 μg for C, and 110 μg for D. The data are shown as micrograms of receptor-active peptide per fraction.

RESULTS

The synthetic gene for hIGF-I was altered to prepare peptides in which (a) the first 16 amino acids of hIGF-I were replaced with the first 17 amino acids of the B-chain of insulin...
IGF-I Analogs with Reduced Affinity for Serum Binding Proteins

FIG. 5. Inhibition of ligand binding to human placental type 1 IGF receptors (left) and insulin receptors (right) by hIGF-I (O), B-chain mutant (O), [Tyr5,Leu6]IGF-I (O), [Gln3,Ala4]-IGF-I (O), and [Glu2,Ala4,Tyr15,Leu16]IGF-I (A). Data are expressed as the percent of maximal specific binding of ligand determined in the absence of added peptide. Each point represents the mean ± S.D. for two determinations.

FIG. 6. Inhibition of ligand binding to human serum binding protein (A) and rat liver type 2 IGF receptor (B) by hIGF-I (O), B-chain mutant (O), [Tyr5,Leu6]IGF-I (O), [Gln3,Ala4]-IGF-I (O), and [Glu2,Ala4,Tyr15,Leu16]IGF-I (A). Data are expressed as the percent of maximal specific binding of ligand determined in the absence of added peptide. Each point represents the mean ± S.D. for two determinations.

(B-chain mutant), (b) the Gln3,Phe16 sequence of hIGF-I was replaced with Tyr-Leu ([Tyr15,Leu16]IGF-I), (c) the Glu3,Thr4 sequence of hIGF-I was replaced with Gln-Ala, ([Gln3,Ala4]-IGF-I), and (d) the 3rd, 4th, 15th, and 16th residues were changed as above [Gln3,Ala4,Tyr15,Leu16]IGF-I. A schematic showing the expression system used to direct synthesis and secretion of the mutant peptides is shown in Fig. 1. Fig. 2 shows the respective DNA sequences of the mutant genes and peptides. Note that in the B-chain mutant a silent codon change for Leu10, CTG→CTC has occurred.

The expression vectors were introduced into a protease-deficient strain of yeast, S. cerevisiae BJ1955, which was grown to saturation in a 1-liter culture. The conditioned media contain receptor active IGF-I-like activity. The peptides were purified by batch treatment with Bio-Rex 70 followed by Bio-Gel P-10 chromatography. The Bio-Gel P-10 elution profile for the B-chain mutant is shown in Fig. 3. The peptides were purified to homogeneity by reverse-phase HPLC (Fig. 4). The peak fractions were pooled and an aliquot analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by colloidal gold protein staining. A single protein band comigrating with [Thr18,Ala19]IGF-I was detected (data not shown).

The final yields of peptide from 1 liter of conditioned media were 230 pg for the B-chain mutant, 131 pg for [Gln3,Ala4]-IGF-I, 162 pg for [Tyr5,Leu6]IGF-I, and 393 pg for [Gln3,Ala4,Tyr15,Leu16]IGF-I.

The peptides all have the correct amino-terminal sequence, except 20% of the B-chain mutant preparation is the des-Phe derivative (data not shown). A minor sequence is also observed in all four preparations suggesting that 5–10% of the preparation has been proteolytically hydrolyzed between Arg27 and Ala28.

The B-chain mutant has 2-fold lower affinity than hIGF-I for the human placental type 1 IGF receptor (Fig. 5, left). [Tyr5,Leu6]IGF-I, [Gln3,Ala4]-IGF-I, and [Gln3,Ala4,Tyr5,Leu6]IGF-I are all equipotent at the insulin receptor. The B-chain mutant, [Tyr5,Leu6]IGF-I, and [Gln3,Ala4,Tyr15,Leu16]IGF-I have 4-, 10-, and 10-fold higher affinity than hIGF-I for the human placental insulin receptor, respectively (Fig. 5, right). [Gln3,Ala4]-IGF-I and hIGF-I are equipotent at the insulin receptor.

The B-chain mutant has dramatically lower affinity for acid-stable human serum binding proteins and the rat liver type 2 IGF receptor (Fig. 6). Concentrations of the B-chain mutant up to 1 μM show little inhibition of ligand binding to acid-stable binding proteins, while hIGF-I inhibits with IC50 ~ 0.48 nM (Fig. 6A). [Tyr5,Leu6]IGF-I and [Gln3,Ala4]IGF-I have 4-fold lower affinity than hIGF-I. However, the peptide with these two mutations combined, [Gln3,Ala4,Tyr15,Leu16]-IGF-I, has 900-fold lower affinity than hIGF-I for acid-stable serum binding proteins (Fig. 6A).

The B-chain mutant at concentrations up to 5 μM does not inhibit binding to the type 2 IGF receptor (Fig. 6B) and, therefore, it has at least 100-fold lower affinity than hIGF-I.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC50 nM</th>
<th>Type 1 IC50 μM</th>
<th>Type 2 IC50 μM</th>
<th>IR IC50 μM</th>
<th>hBP IC50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>5.6 ± 0.8</td>
<td>0.3 ± 0.2</td>
<td>2.8 ± 1.0</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>B-chain mutant</td>
<td>12 ± 1</td>
<td>0.18 ± 0.04</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>[Tyr5,Leu6]IGF-I</td>
<td>4.5 ± 1.5</td>
<td>0.08 ± 0.04</td>
<td>0.3 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>[Gln3,Ala4]-IGF-I</td>
<td>5.3 ± 0.1</td>
<td>1.0 ± 0.5</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.4</td>
<td>300 ± 30</td>
</tr>
<tr>
<td>[Gln3,Ala4,Tyr15,Leu16]IGF-I</td>
<td>5.0 ± 0.1</td>
<td>0.31 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>300 ± 30</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± S.D., n = 2.

1 IR, insulin receptor; hBP, human binding protein.
were neutralized and electrophoresed on a 12% acrylamide gel. Described under "Experimental Procedures." After cross-linking, serum with disuccinimidyl suberate. Ligand alone samples were precipitated with trichloroacetic acid, and the pellets ligand and serum proteins in the absence of hIGF-I, residues -1 to 4, residues 8 and 9, and residues 15 and 16. The high degree of sequence homology between insulin and hIGF-I is the basis of a computer-generated model for the tertiary structure of hIGF-I (21, 22). The model predicts that residues 1-6 have an extended structure, and that residues 8-17 form an α-helix. This model is shown in Fig. 9 along with the model of the B-chain of porcine insulin generated from the X-ray crystal structure data. As can be seen in Fig. 9, the position of the aromatic residue at the bottom of the helix (Phe<sup>6</sup>) is shifted from facing into the hydrophobic core of the peptide in hIGF-I to facing outward into the hydrophilic environment in insulin (Tyr<sup>8</sup>). By analogy with insulin, the aromatic residue of our mutants containing tyrosine at position 15 is also facing outward. Our data show that the dramatic loss of affinity of the B-chain mutant for human serum binding proteins cannot be duplicated by mutation at residues 3 and 4 or at residues 15 and 16 alone. However, [Gln<sup>3</sup>,Ala<sup>4</sup>,Tyr<sup>15</sup>,Leu<sup>16</sup>]IGF-I, the peptide in which all four substitutions are combined, has 600-fold lower affinity for serum binding proteins than hIGF-I. It is not clear whether this indicates that serum binding proteins interact with both the amino-terminal extended region and the residues 15 and 16 at the bottom of the B-chain helix of hIGF-I or if the combined mutation causes a structural change which is unfavorable for binding to the serum binding proteins. The residue corresponding to hIGF-I-Thr<sup>4</sup> is histidine in insulin and the B-chain mutant. Our data suggest that histidine is not required for the loss in affinity to binding protein since substitution of Thr<sup>4</sup> with alanine is effective. Our serum-binding protein data have been generated with a crude acid-treated human serum preparation. Acid treatment is necessary to remove endogenous IGF-I from the binding protein. However, the 150-kDa native binding protein is altered by this treatment. The acid-stable component is 40-43 kDa (7) and is immunologically related to the 150-kDa protein (23). Our cross-linking data show that the major binding component in our preparation is this protein. It is not clear if the binding properties of the native 150-kDa protein are identical to those of the acid-stable 40-kDa protein. However, we have preliminary data suggesting that the B-chain mutant and [Gln<sup>3</sup>,Ala<sup>4</sup>,Tyr<sup>15</sup>,Leu<sup>16</sup>]IGF-I have reduced affinity for the native rat serum binding protein. The minor 38-kDa cross-linked band in our preparation is likely to be the growth hormone-independent species described by Wilkins and D’Ercole (8). This protein is immunologically related to a similar protein purified from amniotic fluid and present in conditioned media of some cell types (24).
FIG. 9. Stereo comparison of the crystal structure of the B-chain of insulin (A) with the proposed structural model of hIGF-I (21, 22) (B).

reported that the binding protein purified from human amniotic fluid enhances the biological response to IGF-I in human fibroblasts. Our data clearly show that the B-chain mutant and [Gln\(^3\),Ala\(^4\),Tyr\(^5\),Leu\(^6\)]IGF-I are fully active in stimulating DNA synthesis in rat A10 cells. The biological potency of these peptides in other in vitro systems and in vivo should help determine the biological function of the IGF binding proteins.

The B-chain mutant has at least 100-fold lower affinity than hIGF-I for the rat liver type 2 IGF receptor. However, [Gln\(^3\),Ala\(^4\)]IGF-I, [Tyr\(^5\),Leu\(^6\)]IGF-I, and [Gln\(^3\),Ala\(^4\),Tyr\(^5\),Leu\(^6\)]IGF-I all have normal affinity for the type 2 receptor. This suggests either that these two regions are not important for maintaining binding or that the more dramatic total change is required. The effect of these mutations on the biological activities of hIGF-I are not clear. At present, there are no known biological functions of IGF-like peptides mediated by the type 2 IGF receptor. However, the B-chain mutant will prove useful in clarifying the biological role of this protein.

The B-chain mutant has a 4-fold increased affinity for the insulin receptor and a 2-fold decreased affinity for the type 1 IGF receptor. The increase in affinity for the insulin receptor seems to require only the change of the Glu\(^3\),Phe\(^{16}\) of hIGF-I to Tyr\(^{15}\),Leu\(^{16}\), since those mutants containing this modification show 10-fold increased affinity. However, [Gln\(^3\),Ala\(^4\)]-IGF-I, [Tyr\(^5\),Leu\(^6\)]IGF-I, and [Gln\(^3\),Ala\(^4\),Tyr\(^5\),Leu\(^6\)]IGF-I all have normal affinity for the type 1 IGF receptor. Thus, the position of the aromatic residue at the bottom of the "B" region helix is not important in type 1 receptor binding. This is an indication that, despite the homology between both the peptide ligands and the membrane receptors, the structural determinants of the ligands which are recognized by the type 1 receptor and the insulin receptor are not always analogous.

In data reported elsewhere, we have shown that deletion of the eight amino acid "D" region of hIGF-I has very little effect on the affinity of hIGF-I for the types 1 or 2 IGF receptors or serum binding proteins (27). We have also shown that substitution of the tyrosine at position 24 of hIGF-I with either leucine or serine causes a 20–30-fold loss in affinity for the type 1 receptor, but no change in the affinity for the type 2 receptor or for human serum binding proteins (28). These data and the data presented in this study provide a detailed analysis of the domains of the "B" and "D" regions of hIGF-I responsible for maintaining high affinity binding to the IGF receptors and serum binding proteins.

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