Insulin and the insulin-like growth factors (IGFs) bind with high affinity to their cognate receptor and with lower affinity to the noncognate receptor. The major structural difference between insulin and the IGFs is that the IGFs are single chain polypeptides containing A-, B-, C-, and D-domains, whereas the insulin molecule contains separate A- and B-chains. The C-domain of IGF-I is critical for high affinity binding to the insulin-like growth factor I receptor, and lack of a C-domain largely explains the low affinity of insulin for the insulin-like growth factor I receptor. It is less clear why the IGFs have lower affinity for the insulin receptor. In this study, 24 insulin analogues and four IGF analogues were expressed and analyzed to explore the role of amino acid differences in the A- and B-domains between insulin and the IGFs in binding affinity for the insulin receptor. Using the information obtained from single substituted analogues, four multiple substituted analogues were produced. A “quadruple insulin” analogue ([Phe\textsubscript{A8}, Ser\textsubscript{A10}, Thr\textsubscript{B5}, Gln\textsubscript{B16}]Ins) showed affinity as IGF-I for the insulin receptor, and a “sextuple insulin” analogue ([Phe\textsubscript{A8}, Ser\textsubscript{A10}, Thr\textsubscript{B5}, Thr\textsubscript{B14}, Gln\textsubscript{B16}]Ins) showed an affinity close to that of IGF-II for the insulin receptor, whereas a “quadruple IGF-I” analogue ([His\textsubscript{4}, Tyr\textsubscript{15}, Thr\textsubscript{A20}, Ile\textsubscript{25}]IGF-I) and a “sextuple IGF-II” analogue ([His\textsubscript{4}, Ala\textsubscript{16}, Tyr\textsubscript{18}, Thr\textsubscript{A20}, Ile\textsubscript{29}, Asn\textsubscript{58}]IGF-II) showed affinities similar to that of insulin for the insulin receptor. The mitogenic potency of these analogues correlated well with the binding properties. Thus, a small number of A- and B-domain substitutions that map to the IGF surface equivalent to the classical binding surface of insulin weaken two hotspots that bind to the insulin receptor site 1.

The structural and functional similarities between insulin and the insulin-like growth factors I and II (IGF-I and IGF-II)\textsuperscript{2} provide a strong indication that their genes share a common evolutionary history (1–3). The biologically active insulin molecule is composed of two polypeptide chains: the A- and B-chain (Fig. 1), which are generated by cleavage from a single chain proinsulin. The A-chain consists of 21 amino acids arranged in two antiparallel α-helices (A1–A8 and A13–A20); the B-chain is composed of 30 amino acids arranged in a central α-helix (B9–B19) with both N and C termini extensions. The two chains are connected by two disulfide bridges (A7-B7 and A20-B19). An additional disulfide bridge is located between residues A6 and A11 in the A-chain (4–6). IGF-I and IGF-II are homologous polypeptides structurally related to insulin. They also consist of an A-domain and B-domain (Fig. 1) with three equivalently located disulfide bridges. In contrast to insulin, the IGFs are single chain polypeptides with an intact C-domain and with an additional D-domain extending from the C-terminal end of the A-domain (7, 8).

The insulin receptor tyrosine kinase family consists of the insulin receptor (IR), insulin-like growth factor I receptor (IGF-IR) and the orphan insulin receptor-related receptor. The members of the insulin receptor family are composed of two receptor halves, each comprising an extracellular α-subunit and a transmembrane β-subunit, linked by a disulfide bridge. The receptors exist as covalent disulfide-linked dimers even when no ligand is bound. The IR and the IGF-IR have sequence similarities varying from 41 to 84%, depending on which regions are being compared (9–11).

Exon 11 of the IR gene is alternatively spliced, resulting in two different transcripts in which 36 additional nucleotides encoding 12 amino acids at the C terminus of the receptor α-subunit are either excluded (IR-A) or included (IR-B) from the mature mRNA (12–14). There is no evidence of such alternative splicing in the IGF-IR gene.

Insulin and the IGFs show similar binding properties for their cognate receptors. All three ligands show curvilinear Scatchard plots and accelerate the dissociation of prebound ligands demonstrating negative cooperativity between two binding sites (15–18). Insulin and the IGFs bind with high affinity to their cognate receptor and with lower affinity for the noncognate receptor. However, the IR-A has been reported to bind IGF-II with high affinity. Therefore, IGF-II might use both the IR and the IGF-IR to exert its effects (19–22).

Lack of C-domain in circulating insulin largely explains why insulin binds the IGF-IR with low affinity (23). It still remains unclear why the IGFs bind the IR with lower affinity. Using chimeric IGF molecules, Denley et al. (20) showed that the
C-domain and, to a lesser extent, the D-domain contribute to the higher affinity of IGF-II than IGF-I for the IR-A and suggested that either domain length or charge differences could be responsible. However, Kristensen et al. (24) made a single chain insulin hybrid containing the C-peptide of IGF-I and showed that the presence of the IGF-I C-peptide had no effect on IR-A binding, whereas increasing the affinity for the IGF-IR to a level close to that of IGF-I. In this paper, we have explored the possibility that sequence differences in the A- and B-domains between insulin and the IGFs are involved in the lower affinity of the IGFs for the IR-A and whether they also contribute to the higher affinity of IGF-II than IGF-I for the IR-A.

By analyzing numerous single substituted insulin analogues, single substituted IGF-I analogues and multiple substituted insulin and IGF analogues, we clearly show that a small number of amino acid differences in the A- and B-chain of insulin and the IGFs are sufficient to explain why the IGFs bind with lower affinity than insulin for the IR-A. Our results also show that there is room for the C-domain of IGF-I and IGF-II in the binding pocket of the IR-A.

EXPERIMENTAL PROCEDURES

Materials—Molecular biology procedures, including agarose gel electrophoresis, restriction enzyme digestion, ligation, bacterial transformation, and DNA sequencing were performed by standard methods. Oligonucleotides were purchased from DNA Technology (Aarhus, Denmark) or Geneworks Pty. Ltd. (Adelaide, Australia). Restriction enzymes and ligase were from New England Biolabs (Hitchin, UK). Recombinant human insulin, A1–B29-insulin, and Achromobacter lyticus protease were from Novo Nordisk A/S (Bagsvaerd, Denmark). Recombinant Long34RhaIGF-I, IGF-I, and IGF-II were purchased from GroPeP Ltd. (Adelaide, Australia). IM9 cells were purchased from ATCC (Middlesex, UK). The L6 rat muscle myoblast cell line (stably transfected with IR-A) was kindly provided by B. F. Hansen (Novo Nordisk A/S, Denmark). Several of the single substituted insulin analogues were a kind gift from S. Havelund (Novo Nordisk A/S, Denmark).

Vector Construction and Yeast Expression of Insulin Analogues—Single substitutions were introduced in the sequence encoding the insulin precursor by QuikChange site-directed mutagenesis (Stratagene). Resultant constructs were subcloned into the yeast expression vector pJB146 (kind gift from Jakob Brandt, Novo Nordisk A/S, Denmark). The yeast expression procedure has been described previously (25). Briefly, an insulin precursor was coupled to a synthetic pre-peptide chain insulin precursor was expressed in the insulin precursor was (B1–B29)-AAK-(A1–A21). The single chain insulin precursor was expressed in Saccharomyces cerevisiae strain MT663. Yeast cells were transformed and selected on yeast nitrogen base, 2% glucose plates. Yeast cultures were grown in yeast/peptone medium with 2% glucose and 50 mM CaCl2 for 72 h at 30 °C (24–26).

Maturation and Purification of the Insulin Precursor—The single chain insulin precursors were converted to des-B30-insulin using a lysine-specific endoprotease (A. lyticus) that removes the spacer peptide and the AAK bridge (27). The pH of the cell-free yeast culture medium with the insulin precursor was adjusted to pH >8.5 with 1 M Tris-HCl (pH 9) and applied to a column with A. lyticus protease immobilized on Sepharose. The column was incubated for 30–60 min at room temperature before the cleaved precursor was eluted from the column with 50 mM Tris-HCl. The insulin analogues were analyzed by mass spectroscopy and were shown to have correct masses. Quantification was performed by LC-MS using human insulin as a standard.

In order to achieve higher concentration, the quadruple and sextuplet substituted insulin analogues were concentrated using a 6 foot2 Millipore Prep/Scale TFF Cartridge, purified using gel filtration and a HiTrap Sepharose HP column (GE Healthcare Ltd.), and finally desalted using a PD-10 column (GE Healthcare).

Vector Construction and Escherichia coli Expression of Human IGF Analogues—The IGF expression vectors were developed by King et al. (28), and the cDNA encoding IGF-I and IGF-II was introduced into the vector as previously described (29). QuikChange site-directed mutagenesis (Stratagene) was used to incorporate the mutations into IGF-I and IGF-II. The resultant constructs were transformed into E. coli JM101 (lac Iq) for expression. IGF mutants were expressed as fusion proteins with the first 11 amino acids of porcine growth hormone ([Met1]pGH-(1–11)) after isopropyl β-D-thiogalactosidase induction. Inclusion bodies were isolated as previously described (28).

Cleavage, Refolding, and Purification of IGF Analogues—Inclusion bodies containing the IGF-I and IGF-II fusion peptides were processed as described in Ref. 29. Briefly, inclusion bodies from 0.5-liter fermentations were solubilized in 8 M urea containing 40 mM glycine, 0.1 M Tris, and 20 mM dithiothreitol (DTT) at pH 9.1 in the case of IGF-I and pH 2.0 for IGF-II. Inclusion bodies were immediately desalted on a Superdex 75 column (1 × 30 cm (Amersham Biosciences) in two 2-ml batches) using the same buffer but with 1.6 mM DTT. Fractions containing the IGF fusion proteins were identified by SDS-PAGE and reverse phase chromatography and pooled prior to folding. IGF-I folding was performed by dilution in 2 M urea, 10 mM glycine, 0.1 M Tris, 5 mM EDTA, 0.4 mM DTT, 1 mM 2-hydroxyethyl disulfide, pH 9.1, to less than 0.1 mg/ml. IGF-II folding was performed by dilution in 2.5 M urea, 12.5 mM glycine, 0.7 M Tris, 5 mM EDTA, 0.4 mM DTT, 1 mM 2-hydroxyethyl disulfide, pH 9.1, to less than 0.1 mg/ml. Cleavage of the fusion partner and a final reverse phase HPLC cleanup were achieved as previously described (30, 31). Purified IGF analogues were analyzed by mass spectroscopy (Dr. Chris Bagley and Chris Cursaro, Adelaide Proteomics Facility) and were shown to have the correct masses. Quantification of analogues was performed by comparing analytical C4 high pressure liquid chromatography profiles with profiles of standard Long34RhaIGF-I preparations (32).

Binding Assays—Whole cell receptor binding assays were performed as previously described (15) using IM9 cells, which show high expression of the IR-A (~20,000 binding sites/cell). For competition assays, 2.5 × 106 cells/ml were incubated with 125I-insulin (20,000 cpm) in the presence of increasing concentrations of the analogue in a final volume of 500 μl for 2.5 h at
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**FIGURE 1. Sequence alignment and domain organization of insulin, IGF-I, and IGF-II.** The conserved regions between insulin, IGF-I, and IGF-II are highlighted in light gray boxes, and conserved regions between IGF-I and IGF-II are highlighted in dark gray boxes. Insulin residues mutated in this and previous studies are indicated (boldface type), and IGF-I and IGF-II residues that have a ≥2-fold change in affinity when introduced in insulin are also indicated (boldface type). Residues that are further examined in this study are highlighted (asterisk).

**TABLE 1** Competitive binding for the IR-A of increasing concentrations of single substituted insulin analogues and a fixed concentration of 125I-insulin

| Residue in | Insulin | IGF-I | IGF-II |
|-----------|---------|-------|--------|
|           | Insulin | IGF-I | IGF-II |
|           | Kd ± S.D. | n | Rel Kd |
| —         | —       | — | — |
| GluA12        | Glu    | Glu    | Glu    |
| ArgA6       | Arg    | Arg    | Arg    |
| SerA16       | Ser    | Ser    | Ser    |
| AspA12       | Asp    | Asp    | Asp    |
| AlaA10       | Ala    | Thr    | Ser    |
| ArgA15       | Glu    | Arg    | Leu    |
| LeuA15       | Glu    | Leu    | Thr    |
| ThrA18       | Thr    | Thr    | Thr    |
| AlaA21       | Asn    | Asn    | Asn    |
| GlyA22       | Gly    | Val    | Ser    |
| ProA22       | Pro    | Val    | Pro    |
| AsxA14       | Asx    | Asx    | Asx    |
| SerA13       | Ser    | Ser    | Ser    |
| GluA21       | Glu    | Glu    | Glu    |
| ThrB5        | Thr    | His    | Thr    |
| AlaB19       | Ala    | Ser    | Ala    |
| GlyB19       | Ser    | Gly    | Ser    |
| GluB10       | His    | Gly    | Glu    |
| ThrB14       | Ala    | Thr    | Thr    |
| GlyB16       | Tyr    | Gly    | Gly    |
| PheB17       | Leu    | Phe    | Phe    |
| TyrB25       | Phe    | Tyr    | Tyr    |
| PheB26       | Tyr    | Phe    | Phe    |
| SerB27       | Thr    | Ser    | Ser    |
| ArgB28       | Pro    | Arg    | Arg    |

* Data from Ref. 55.
* Data from Ref. 56.
* Data from Ref. 35.

15 °C in Hepes binding buffer (100 mM Hepes, 100 mM NaCl, 5 mM KCl, 1.3 mM MgSO4, 1 mM EDTA, 10 mM glucose, 15 mM sodium acetate, 1% bovine serum albumin (w/v), pH 7.6). Afterward, 200 μl of the mixture was centrifuged at 14,000 rpm for 5 min. The cell pellet was counted in a Wallac WIZARD γ counter (PerkinElmer Life Sciences). The assays were done at least three times in duplicate for each analogue. Binding data were corrected for nonspecific binding and analyzed by computer fitting to a one-site model, using an Excel program developed in our laboratory by A. V. Groth and R. M. Shymko, to obtain the dissociation constant (Kd).

For dose-response curves for negative cooperativity (accelerated dissociation) (15), 5 × 10⁶ cells/ml were incubated with 125I-insulin (150,000 cpm/ml of cells) for 2 h at 15 °C. Afterward, the cells were centrifuged, and the supernatant (unbound ligand) was removed. The cell pellet was resuspended to the same concentration in Hepes binding buffer, and 25 μl of cells were transferred to tubes containing increasing concentrations of cold analogue in 1 ml of Hepes binding buffer at 15 °C. The dissociation of prebound ligand was stopped after 30 min by centrifugation at 4000 rpm for 5 min. The cell pellet was counted in the γ counter. Each concentration was measured in duplicate, and assays were repeated at least three times.

[^3]: C. Bonnesen, and M. B. Oleksiewicz. manuscript in preparation.
2-fold change as a cut-off is based on alanine-scanning mutagenesis studies, which have demonstrated that any meaningful change in affinity, produced by a single substitution, ranges from 2- to 100-fold (33, 34).

The analogue that showed the lowest affinity was [GlnB16]Ins. Replacing tyrosine with glutamine had a large impact on binding, leading to an almost 10-fold decrease in affinity. Introducing serine instead of isoleucine in position A10 and threonine instead of histidine in position B5 resulted in a 4-5-fold reduction in affinity, and finally introduction of phenylalanine instead of threonine in position A8 and instead of tyrosine in position B26 resulted in a 2-fold reduction in affinity. All five substitutions were amino acids present in both IGF-I and IGF-II; therefore, no IGF-I- or IGF-II-specific substitutions resulted in a decrease in affinity.

Three substitutions resulted in an increase in affinity compared with that seen of human insulin (Table 1 and Fig. 2). Those were the already known [GluB10]Ins (35) and two novel analogues: [ThrA18]Ins and [ThrB14]Ins. Interestingly, these two residues differ only in IGF-II.

When analyzing the location of the residues that have an effect on binding (Fig. 2), it becomes clear that ThrA8, IleA10, and HisB5 constitute a small connected area on the surface of the insulin molecule and could combine to play a role in achieving high affinity binding of insulin to the IR.

Production, Quantification, and Binding of the Multiple Substituted Insulin Analogues—Having established that only five substitutions resulted in a considerable decrease in affinity, seven new insulin analogues combining three or four of these substitutions were produced with the aim to generate an insulin molecule with a binding affinity equivalent to IGF-I. Since [PheA8]Ins and [PheB26]Ins only showed a borderline 2-fold reduction in affinity compared with human insulin, these two could have been excluded in further analysis. However, since ThrA8 is located next to the IleA10 and HisB5 on the surface of the insulin molecule, the PheA8 substitution was included in the new analogues.

Heterologous competition assays were again performed on the IR-A to determine any changes in affinity of the analogues with multiple substitutions compared with human insulin. As illustrated in Table 2, all analogues showed a clear reduction in affinity compared with human insulin. Including GlnB16 in the triple mutated insulin analogues ([PheA8,SerA10,GlnB16]Ins and [SerA10,ThrB5,GlnB16]Ins) resulted in a marked decrease in
ThrB5, GlnB16]Ins) resulted in a 150-fold decrease in affinity.

Interestingly, this affinity was almost identical to that seen of IGF-I when competing with 125I-insulin for the IR-A on the IM9 cells (Fig. 3B). Therefore, combining all six substitutions into insulin results in an affinity similar to that of IGF-I.

Next, we aimed to produce an insulin molecule with an IR-A binding affinity equal to IGF-II. We made two quintuple substituted insulins ([PheA8, SerA10, ThrA18, ThrB5, GlnB16]Ins and [PheA8, SerA10, ThrB5, ThrB14, GlnB16]Ins) and a sextuple substituted insulin ([PheA8, SerA10, ThrA18, ThrB5, ThrB14, GlnB16]Ins) that incorporated the two substitutions that were in residues differing only between insulin and IGF-II ([ThrA18]Ins and [ThrB14]Ins). Comparing the binding affinities of the two insulin analogues with five substitutions ([PheA8, SerA10, ThrA18, ThrB5, ThrB14, GlnB16]Ins and [PheA8, SerA10, ThrB5, ThrB14, GlnB16]Ins) confirmed that especially threonine in position B14 plays a positive role in binding to the IR-A, leading to a higher affinity (Table 2). The [PheA8, SerA10, ThrA18, ThrB5, ThrB14, GlnB16]Ins (for simplicity called “sextuple insulin”) resulted in a higher affinity than IGF-I, very similar to that seen of IGF-II when competing with 125I-insulin for the IR-A on the IM9 cells (Fig. 3B). Therefore, combining all six substitutions, four substitutions that show a decrease in affinity and two IGF-II specific substitutions that show an increase in affinity, resulted in an affinity similar to the affinity of IGF-II.

Production, Quantification, and Binding of Single and Multiple Substituted IGF Analogues—We used a similar approach to determine whether we could make IGF-I or IGF-II analogues that behave like insulin. Four single amino acid substitutions and a quadruple substitution into IGF-I and a sextuple substitution into IGF-II were made using the reciprocal insulin amino acid substitutions. All six analogues were expressed successfully in E. coli. Mass spectrometry and IGF-IR binding were used as indicators of correct purification and folding of each analogue (data not shown). None of the analogues showed significant changes in affinity for the IGF-IR (data not shown).

Again, heterologous competition assays were performed to determine any changes in affinity for the IR-A upon multiple substitution compared with human insulin, IGF-I, and IGF-II. The average Kd value ± S.D. for each analogue is shown in Table 3 together with insulin, IGF-I, and IGF-II. By introducing into IGF-I the insulin residues homologous to the

| Substance | Kd (nM) | n | Rel Kd |
|-----------|---------|---|--------|
| Insulin   | 0.3 ± 0.1 | 22 | 1      |
| [PheA8, SerA10, ThrA18, ThrB5]Ins | 5.0 ± 0.3 | 3 | 17.3  |
| [PheA8, SerA10, GlnB16]Ins | 22.1 ± 1.5 | 3 | 76.2  |
| [SerA10, ThrB5, GlnB16]Ins | 20.9 ± 1.9 | 3 | 72.1  |
| [PheA8, SerA10, ThrB5, GlnB16]Ins quadruple insulin | 43.3 ± 4.5 | 3 | 149.3 |
| [PheA8, SerA10, ThrA18, ThrB5, GlnB16]Ins | 30.9 ± 2.6 | 3 | 106.6 |
| [PheA8, SerA10, ThrA18, ThrB5, ThrB14, GlnB16]Ins | 15.1 ± 0.4 | 3 | 52.1  |
| [PheA8, SerA10, ThrA18, ThrB5, ThrB14, GlnB16]Ins sextuple insulin | 10.6 ± 1.0 | 3 | 36.6  |
| IGF-I     | 49.7 ± 7.2 | 3 | 171.4 |
| IGF-II    | 6.1 ± 0.4 | 3 | 21.0  |

FIGURE 3. Quadruple insulin and sextuple insulin binding to the IR-A. Binding curves showing the competing effect of insulin (○), IGF-I (●), and quadruple insulin (■) (A) and insulin (×), IGF-II (□), and sextuple insulin (□) (B) for the IR-A. Increasing concentrations of the ligand compete with a fixed concentration of 125I-insulin for the IR-A using the IM9 cell line. Curves are illustrated as specific binding/specific binding at 0M cold ligand and are representative of three assays, each made in duplicate. S.D. values are shown for all of the data (smaller than the symbol when not visible).
IGF residues that lead to a decrease in affinity when mutated in insulin, we expected to affect the affinity for the IR in a "positive" manner, leading to an increase in affinity compared with IGF-I. [His4]IGF-I and [Tyr15]IGF-I (equivalent to [ThrB5]Ins and [GlnB16]Ins) indeed resulted in a clear increase in affinity compared with that of IGF-I, whereas [Thr49]IGF-I and [Ile51]IGF-I (equivalent to [PheA8]Ins and [SerA10]Ins) showed no clear changes in affinity compared with IGF-I (Table 3).

Combining all four substitutions into a quadruple substituted IGF-I analogue ([His4,Tyr15,Thr49,Ile51]IGF-I) had a very striking effect on affinity, since this analogue has an affinity very close to that of insulin for the IR-A (Fig. 4). Thus, simply introducing four insulin substitutions into IGF-I is enough to achieve insulin-like affinity for the IR-A (this analogue will for simplicity be called "quadruple IGF-I").

Finally, a sextuple substituted IGF-II analogue ([His7,Ala16,Tyr18,Thr48,Ile50,Asn58]IGF-II) was analyzed in order to see what effect these substitutions had on IGF-II binding to the IR-A (Fig. 4B). This analogue (for simplicity called "sextuple IGF-II") showed a small increase in affinity compared with that of IGF-II (Table 3) but still had a poorer affinity than insulin and the quadruple substituted IGF-I analogue.

**Dose-Response Curves for Negative Cooperativity**—In order to further validate the binding affinities, we tested whether quadruple insulin, sextuple insulin, quadruple IGF-I, and sextuple IGF-II were able to accelerate dissociation of prebound 125I-insulin (Fig. 5).

Quadruple insulin and sextuple insulin were both very poor at accelerating the dissociation of prebound 125I-insulin compared with human insulin and also compared with IGF-I and IGF-II (Fig. 5, A and B). In contrast, quadruple IGF-I and sextuple IGF-II were able to accelerate dissociation of prebound 125I-insulin to the same degree as human insulin (Fig. 5, C and D).

**Mitogenic Potency**—Finally, to see whether the effects of the multiple substitutions on affinity were translated into effects on biological potencies, the mitogenic potencies of the analogues were tested. As illustrated in Fig. 6, the mitogenic potencies correlated well with the dose-response curves for accelerated dissociation.

Quadruple insulin and sextuple insulin were both poor inducers of mitogenicity compared with insulin and with IGF-I and IGF-II (Fig. 6A), whereas quadruple IGF-I and sextuple IGF-II were fully able to induce mitogenicity to a similar degree as insulin and more potently than IGF-I and IGF-II through the IR-A (Fig. 6, B and C).

### TABLE 3

| Residue in | IGF-I | Insulin | IGF-II | \(K_d \) ± S.D | n | \(Rel \ K_d \) |
|-----------|-------|---------|--------|---------------|---|----------|
| [His4]IGF-I | Thr | His | Thr | 0.3 ± 0.1 | 22 | 1 |
| [Tyr15]IGF-I | Gln | Tyr | Gln | 12.2 ± 2.6 | 3 | 42.1 |
| [Thr49]IGF-I | Phe | Thr | Phe | 13.0 ± 3.6 | 3 | 44.8 |
| [Ile51]IGF-I | Ser | Ile | Ser | 47.6 ± 9.9 | 3 | 164.1 |
| [His4,Tyr15,Thr49,Ile51]IGF-I quadruple IGF-I | | | | 81.4 ± 12.1 | 3 | 279.7 |
| [His7,Ala16,Tyr18,Thr48,Ile50,Asn58]IGF-II sextuple IGF-II | | | | 1.2 ± 0.2 | 4 | 4.1 |
| IGF-I | | | | 2.3 ± 0.2 | 3 | 7.9 |
| IGF-II | | | | 49.7 ± 7.2 | 3 | 171.4 |
| | | | | 6.1 ± 0.4 | 3 | 21.0 |

**FIGURE 4.** Quadruple IGF-I and sextuple IGF-II binding to the IR-A. Binding curves showing the competing effect of insulin (×), IGF-I (○), and quadruple IGF-I (△) (A) and insulin (×), IGF-II (○), and sextuple IGF-II (△) (B) for the IR-A. Increasing concentrations of the ligand compete with a fixed concentration of 125I-insulin for the IR-A using the IM9 cell line. Curves are illustrated as specific binding/specific binding at 0 nM cold ligand and are representative of three assays, each made in duplicate. S.D. values are shown for all of the data (smaller than the symbol when not visible).
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**FIGURE 5.** Dose-response curves for negative cooperativity (quadruple insulin, sextuple insulin, quadruple IGF-I, and sextuple IGF-II). Dissociation of prebound $^{125}$I-insulin in the presence of increasing concentrations of insulin (×) is illustrated in all four curves. Dissociation with increasing concentration of IGF-I (■) and quadruple insulin (▲) and quadruple IGF-I (▲) (A), IGF-II (▲) and sextuple insulin (□) (B), IGF-I (■) and quadruple IGF-I (▲) (C), and IGF-II (▲) and sextuple IGF-II (□) (D). Curves are illustrated as bound/bound at 0 M cold ligand after 30 min of dissociation. The curves are an average of three assays, each made in duplicate. S.D. values are shown for all of the data (smaller than the symbol when not visible).

**DISCUSSION**

The structures of insulin and the IGFs and their receptors have been extensively studied over the last decades, but there are still many unanswered questions regarding structure-function relationship. Insulin and the IGFs as well as their receptors are very similar in both primary and three-dimensional structures but still bind to the noncognate receptor with much reduced affinity.

The mechanism and structure-function relationships of insulin binding to the IR have been investigated in great detail (for a review, see Refs. 10, 16, and 17). A large body of evidence supports the concept that the insulin molecule comprises two separate binding surfaces, site 1 and site 2, that upon binding cross-link two distinct binding sites (sites 1 and 2) on the α subunits of each IR half-dimer (10, 16–18), thereby creating high affinity binding of one insulin molecule within the IR dimer. Site 1 of insulin is known as the “classical binding surface,” since it was described over 3 decades ago (36, 37). It overlaps with the dimerization interface of insulin and comprises amino acids GlyA1, IleA2, ValA3, GlnA5, TyrA19, AsnA21, ValB12, TyrB16, GlyB23, PheB24, PheB25, and TyrB26. Site 2 of insulin was mapped more recently by alanine mutagenesis of insulin using high affinity binding to IM-9 cells (17); it overlaps with the hexamerization surface of insulin and comprises amino acids SerA12, LeuA13, GlnA17, HisB10, GlnB13, and LeuB17. A variety of biochemical and mutagenesis data, supported by the recent structure of the IR extracellular domain (10, 17, 38, 39, 40), have converged to suggest that the receptor site 1 comprises the N-terminal L1 receptor domain together with a short peptide fragment of the FnIII-2 insert (of unknown structure), probably cooperating in trans between the two α subunits (18), whereas site 2 probably comprises surface residues of FnIII.

The residues of IGF-I involved in IGF-IR binding have not been as completely mapped as for insulin binding to the IR. A number of residues in IGF-I that are homologous to key residues of the insulin classical binding surface have also been shown to be critical for IGF-I binding to the IGF-IR (i.e. TyrA19, Ala62, PheB23, and TyrB24, corresponding to TyrA19, AsnA21, PheB24 and PheB25 of insulin) (31, 41, 42). In particular, the naturally occurring IGF-I mutation V44M found in a Dutch dwarf patient has a significant reduction in IGF-IR binding affinity compared with IGF-I (43, 44). This position is equivalent to ValA3 in insulin, which is believed to be part of the classical binding surface and which is mutated in insulin Wakayama (45, 46). In addition, several residues appear to be unique for IGF-I binding, such as Ala8, Tyr31, Arg36, Arg37, and Met59 (9, 31, 41, 47). In the case of IGF-II binding, Phe26, Tyr27, and Val43 have been shown to be important for binding to the IGF-IR, again corresponding to insulin PheB24, PheB25, and ValA3 (48).

Whether it is the same residues or different residues in IGF-I and IGF-II compared with insulin that are involved in binding to the IR is still unknown. However, the Val44 IGF-I, the Tyr24 IGF-I, and the Tyr60 IGF-I mutants also showed a marked decrease in binding to the IR, indicating that some of the residues involved in insulin binding to the IR could be the same for the IGFs (41, 43).

In order to investigate the IGF A- and B-domain substitutions that weaken IGF binding to the IR-A, a series of 24 insulin analogues with single substitutions were produced, and their binding affinity for the IR-A was analyzed. Most of the analogues showed no changes in affinity, which was not unexpected due to the high homology between insulin and the IGFs. The results obtained in this study, together with previous data obtained, have now led to an almost complete picture of the different contributions of single residue substitutions found in IGF-I and/or IGF-II responsible for the decreased affinity of the IGFs (Table 1).

When looking at the location of the residues with decreased affinity in relation to the two binding surfaces, it is interesting that IleA10 and HisB5 cluster around the A8 position (Fig. 2). This position is believed to introduce favorable and unfavorable interactions at the edge of the IR and has recently been analyzed in regard to receptor binding (49). Analysis of a photoactive A8 analogue showed that it exhibited efficient cross-linking to the insert in the second fibronectin type III domain of the IR that
cooperates (probably in trans (50)) with the L1 domain to create binding site 1.

In a similar cross-linking study, a photoactive B16 analogue demonstrated contact with the L1/H9252 helix of the IR, a region already known to confer high affinity insulin binding (51) and also part of binding site 1. When combining this with the results obtained in this study, it is clear that TyrB16 is important for high affinity binding to the IR. The affinities obtained in this study are consistent with previous results from Schwartz et al. (52), who showed that this substitution displayed only 9% of the potency of insulin in binding to the IR from rat liver plasma membranes. Mutation of TyrB16 into phenylalanine or tryptophan only showed a modest change in IR affinity (53). It therefore appears that the existence of an aromatic ring in this position is important for high affinity binding to the IR, and therefore, lack of this aromatic ring together with unfavorable interactions in position A8 or the neighboring A10 and B5 could explain the lower affinity of the IGFs for the IR-A by weakening two critical attachment spots of the “classical binding surface” to the receptor site 1.

This hypothesis was further supported by combining the four substitutions leading to reduced affinity for IR-A. Quadruple insulin showed very low affinity for the IR. The low affinity was further supported by a lack of accelerated dissociation of prebound 125I-insulin and low mitogenic potency. Combining these four substitutions with the two IGF-II-specific residues that increase the affinity for the IR-A, sextuple insulin, resulted in an analogue with higher affinity compared with quadruple insulin. This analogue also showed improved mitogenic potency compared with quadruple insulin, however only to a small degree. The threonine in position 16 in IGF-II (B14 in insulin) has recently also been shown to be important for high affinity binding of IGF-II to the cation-independent mannose 6-phosphate receptor to which insulin and IGF-I do not bind (54); therefore, this position seems to be important for IGF-II interaction with receptors.

The role of the residues found by scanning insulin in respect to IR binding was fully supported by analyzing two IGF analogues bearing reciprocal substitutions. Quadruple IGF-I showed an affinity very close to that of insulin (only 4-fold lower), and this analogue was able to accelerate dissociation of prebound 125I-insulin with the same potency as insulin. The mitogenic potency of this analogue was also fully comparable with that seen of insulin. Sextuple IGF-II also showed an increase in affinity compared with IGF-II for the IR-A (7.9-fold lower than insulin). However, again this analogue was fully competent in accelerating the dissociation of prebound 125I-insulin and in inducing mitogenic potency. The lower affinity of the sextuple IGF-II compared with the quadruple IGF-I is probably due to the two extra substitutions (T16A and T58N) in the former, given that single substitution in insulin at these positions with the equivalent IGF-II residues led to an increase in affinity for the IR-A (Table 1). It might be interesting to test how this specificity is affected by the presence of the C-domain of IGF-I and IGF-II.

Interestingly, IGF-I and IGF-II were more potent at inducing accelerated dissociation of prebound 125I-insulin and mitogenic potency compared with quadruple insulin and sextuple insulin (Figs. 5 and 6) despite their ability to bind with similar affinities as IGF-I and IGF-II, respectively. This would suggest that although the affinities are similar, there are subtle differences in local contacts made between the IGFs and the insulin analogues that contribute to the interaction with the IR-A.

It should be noted that of the six substitutions studied in this work, only Phe49 has been shown to play a role in binding to the...
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IGF-binding proteins (reviewed in Ref. 11). Mutation of Phe19 to alanine decreased IGF-I binding to IGFBP-1 80,000-fold and to IGFBP-3 15-fold. It is not known what effect the mutation to threonine reported here has on IGFBP binding. We do not think that the IGFBPs interfere in any way in our assays, since the cells are carefully washed before the binding assay, the IGF analogues show no changes in affinity for the IGF-IR, and IGF-II achieves a relatively high binding to the IR-A.

In summary, we have shown that it is possible to produce an IGF-I analogue that binds with a similar affinity as insulin for the IR-A simply by introducing four insulin residues. This suggests that the C-domain can be accommodated within the IR-A binding pocket. These results support the observations made by C. Kristensen et al. (24) that a single chain insulin analogue (A- and B-chain connected by the C-domain of IGF-I) binds with the same affinity as insulin for the IR-A. It would also suggest that in the study by Denley et al. (20), differences in the C-domain side chain properties rather than size influence the relative binding affinities of IGF-I and IGF-II for the IR-A. Lack of the Thr314, Ile315, His316, and Tyr316 contact points could explain why the IGFs bind with lower affinity to the IR-A, since introducing these residues into the IGFs results in affinity and potency very close to that seen of insulin.

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