**Alanine Scanning of a Putative Receptor Binding Surface of Insulin-like Growth Factor-I**

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Current evidence supports a binding model in which the insulin molecule contains two binding surfaces, site 1 and site 2, which contact the two halves of the insulin receptor. The interaction of these two surfaces with the insulin receptor results in a high affinity cross-linking of the two receptor α subunits and leads to receptor activation. Evidence suggests that insulin-like growth factor-I (IGF-I) may activate the IGF-I receptor in a similar mode. So far IGF-I residues structurally corresponding to the residues of the insulin site 1 together with residues in the C-domain of IGF-I have been found to be important for binding of IGF-I to the IGF-I receptor (e.g. Phe\(^{13}\), Tyr\(^{24}\), Tyr\(^{31}\), Arg\(^{66}\), Arg\(^{37}\), Val\(^{44}\), Tyr\(^{60}\), and Ala\(^{62}\)). However, an IGF-I second binding surface similar to site 2 of insulin has not been identified yet.

In this study, we have analyzed whether IGF-I residues corresponding to the six residues of the insulin site 2 have a role in regulating growth and development (1, 2). IGF-I consists of 70 amino acid residues arranged in four domains. The A and B domains are homologous to the A and B chains of insulin (Fig. 1), whereas the C-domain connecting the A- and B-domain and the D-domain extending from the C-terminal end of the A-domain are only present in IGF-I (3–5). The three-dimensional structures of insulin and IGF-I are also very similar. The B-domain of the two molecules is arranged in a central α-helix including residues 8–17 (B\(^9\)–B\(^{19}\)) (insulin residues in parentheses), and the A-domain contains two anti-parallel α-helices including residues 43–48 (A\(^{1}\)–A\(^{8}\)) and residues 54–60 (A\(^{13}\)–A\(^{20}\)) (4, 6, 7).

The insulin receptor family consists of the insulin receptor (IR), insulin-like growth factor-I receptor (IGF-IR) and the insulin-receptor-related receptor, all of which are receptor-tyrosine kinases. The members of the insulin receptor family consist of two receptor halves, each comprising an extracellular α-subunit and a transmembrane β-subunit, linked by a disulfide bridge. The receptor exists as a dimer when no ligand is bound, making an α2β2 receptor held together by disulfide bridges between the two α-subunits. The IR and the IGF-IR have sequence similarities varying from 41 to 84% depending on which regions are being compared. The overall structure of the L1–Cys-rich–L2 domains of the two receptors have been shown by x-ray crystallography to be similar (8–11).

Numerous studies indicate that the IR and IGF-IR contains two separate binding sites for the ligand; however, only one ligand binds and activates the α2β2 receptor dimer. Insulin is believed to interact with the receptor using two binding surfaces, which cross-link the two receptor halves. This cross-linking is important for high affinity binding and thereby for downstream signaling (9, 12–14). The site 1 binding surface of insulin encompasses almost all of the dimerization surface of insulin, whereas the site 2 binding surface is located on the opposite side of insulin and includes several residues also involved in the hexamer-forming surface of insulin (12, 15, 16). IGF-I always exists as a monomer in solution having no dimer- and hexamer-forming surfaces. Therefore, the question arises of whether IGF-I interacts with the receptor in a similar “two-surface” manner as insulin and whether the surfaces are equivalent to the binding surfaces of insulin.

A large body of evidence suggests that IGF-I activates the IGF-IR in a similar way as insulin. IGF-I binding to the IGF-IR has been analyzed extensively and shows similar binding properties to those seen for insulin (12, 13, 17–19). IGF-I binding exhibits a curvilinear Scatchard plot indicating the co-existence of more than one binding site. The dis-
Association of prebound labeled IGF-I is accelerated in the presence of increasing concentration of cold ligand, indicating that the binding sites exert negative cooperativity (17, 19). The main difference in binding properties between IGF-I and insulin is that the dose-response curve for negative cooperativity is sigmoid rather than bell-shaped. Unlike insulin, there is no loss of accelerated dissociation at high concentrations of IGF-I.

Several IGF-I residues equivalent to the residues believed to compose the site 1 binding surface of insulin are important for binding of IGF-I to the IGF-IR, i.e. Phe23 (PheB24), Tyr24 (PheB25), Val44 (ValA3), Tyr60 (TyrA19), and Ala62 (AsnA21) (insulin residues are in parentheses) (8, 20–23). In addition, several IGF-I residues are involved in IGF-IR binding, including Ala8, Met59, and some C-domain residues, i.e. Tyr31, Arg36, and Arg37, but are not important for insulin binding to its receptor (because there is no C-domain is present in insulin) (8, 21, 22, 24, 25).

A computational model of IGF-I docked into one receptor half of IGF-IR (26) (see Fig. 2A) illustrates a way in which IGF-I could interact with one receptor half. In this model the IGF-I molecule is docked using a few parameters (Asp8 and Glu90 on the receptor <4.5 Å from IGF-I, Tyr24 of IGF-I <4.5 Å from the IGF-IR, and finally, Arg36 and Arg37 <8 Å from Glu242 on the IGF-IR). Fig. 2A illustrates this model, with residues corresponding to the site 2 binding surface of insulin highlighted in black, i.e. Glu9 (HisB10), Asp12 (GluB13), Phe16 (LeuB17), Asp53 (SerA12), Leu54 (LeuA13), and Glu58 (GluA17) (insulin residues in parentheses) (Fig. 2, B and C) (12, 26). It is clear from Fig. 2 that a surface more or less equivalent to the second binding surface of insulin is exposed and could have a role in cross-linking to the opposite receptor half. Previously IGF-IR binding analysis of Asp12 to alanine substitution and a Glu9 to lysine substitution suggested that these residues...
are important for IGF-IR binding (27, 28). However, a comparative study of all candidate site 2 residues has never been made.

Here we show by alanine scanning mutagenesis that several of the IGF-1 residues corresponding to the putative second binding surface of insulin are important for achieving high affinity IGF-I binding to the IGF-IR. The data show that Glu9 together with Asp27, Phe16, Leu34, and Glu58 play an important role in high affinity binding of IGF-I to the IGF-IR.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Lines**—Molecular biological procedures, including agarose gel electrophoresis, restriction enzyme digestion, ligation, bacterial transformation, and DNA sequencing were performed by standard methods. Oligonucleotides were purchased from Geneworks Pty Ltd (Adelaide, South Australia). Restriction enzymes and ligase were from New England Biolabs (Hitchin, UK). Recombinant LongTR3IGF-I and IGF-I were purchased from ProPeptide (Adelaide, South Australia). Europium-labeled IGF-I was produced as described by Denley et al. (29) according to the manufacturer’s instructions.125I-labeled IGF-I was made at Novo Nordisk A/S, Denmark.

Human embryonic renal cells (293EBNA) transfected with cDNA encoding full-length human IGF-IR (293EBNA IGF-IR) were made as previously described (30). The P6 BALB/c3T3 cell line overexpressing the IGF-IR (P6 IGF-IR) was a kind gift from Professor R. Baserga (Philadelphia, PA). The L6 rat muscle cell line was kindly provided by M. Oleksiewicz (Novo Nordisk A/S, Denmark).

**Vector Construction and Escherichia coli Expression of Human IGF-I Analogues**—The IGF expression vector was developed by King et al. (31), and the cDNA encoding IGF-I was introduced into the vector as previously described (22, 32). QuikChange site-directed mutagenesis (Stratagene) was used to incorporate the mutations into IGF-I. Resultant constructs were transformed into E. coli JM101 (lacIq) for expression. IGF mutants were expressed as fusion proteins with the first 11 amino acids of porcine growth hormone ((Met 1) pGH (1–11)) after isopropyl β-D-thiogalactoside induction. Inclusion bodies were isolated as previously described (31).

**Refolding, Cleavage, and Purification of IGF-I Analogues**—Inclusion bodies containing the IGF-I fusion peptides were processed as described in Shooter et al. (22) and Francis et al. (32). Briefly, inclusion bodies from a 0.5-liter fermentation were dissolved in 8 M urea containing 40 mM glycine, 0.1 M Tris, and 20 mM dithiothreitol at pH 9.1. Inclusion bodies were immediately desalted on a Superdex 75 column (1 × 30 cm (GE Healthcare)) using the same buffer but with 1.6 mM dithiothreitol. Fractions containing the IGF-I fusion proteins were identified by SDS-PAGE and by reverse-phase chromatography and pooled. IGF-I folding was performed in 2 M urea, 10 mM glycine, 0.1 M Tris, 5 mM EDTA, 0.4 mM dithiothreitol, 1 mM 2-hydroxyethyl disulfide, pH 9.1, and by dilution to less than 0.1 mg/ml. Cleavage of the fusion partner and a final reverse phase HPLC clean up were achieved as previously described (22, 33). Purified IGF-I analogues were analyzed by mass spectroscopy (Dr. Chris Bagley and Chris Cursaro, Adelaide Proteomics Facility) and were shown to have correct masses. Quantification of analogues was performed by comparing analytical C4 HPLC profiles with profiles of standard LongTR3IGF-I preparations. Correct folding of the analogues was tested by recording far UV circular dichroism (CD) spectra. This method has been described previously (34).

**Binding Assays**—Receptor affinities were measured using two different approaches; that is, a soluble receptor assay and a whole cell receptor assay. The soluble receptor assay was performed as described in Delaine et al. (34). Briefly, P6 IGF-IR cells were serum-starved 4 h before lysis with lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5) for 1 h at 4 °C. Lysates were centrifuged for 10 min at 4000 rpm at 4 °C, and 100-μl aliquots were added to each well (white Greiner Lumitrac 600 plate coated with anti-IGF-I antibody 24-31 (2.5 μg/ml)) (35). Approximately 500,000 fluorescence counts of europium-labeled IGF-I were added to each well together with increasing concentrations of unlabeled ligand and incubated overnight at 4 °C. Wells were washed 4 times with 20 mM Tris, 150 mM NaCl, and 0.1% (v/v) Tween 20 and twice with water. DELFIAl enhancement solution (Perkin-Elmer Life Sciences) (100 μl/well) was added before time-resolved fluorescence was measured using 340-nm excitation and 612-nm emission filters with a BMG Laboratory Technologies Polarstar fluorimeter (Mornington, Australia). The assays were performed at least three times for each analogue. EC50 values were calculated using a sigmoid dose-response curve fit in the program GraphPad Prism 4.0.

Whole cell receptor binding assays were performed using 293EBNA IGF-IR cells. For competition assays 1.0 × 105 cells/ml (10–20% binding) were incubated with 125I-labeled IGF-I (20,000 cpm/50 μl) in the presence of increasing concentrations of the cold ligand in a final volume of 500 μl for 3 h at 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, two separate 200-μl aliquots were transferred to centrifuge tubes and centrifuged at 14,000 rpm for 5 min. The membrane pellet was counted in a Wallac WIZARD gamma counter (Perkin-Elmer Life Sciences). The assays were performed at least three times for each analogue. Binding data were corrected for nonspecific binding and analyzed by computer-fitting using an Excel program developed in our laboratory by A. V. Groth and R. M. Shymko to obtain the equilibrium dissociation constant (Kd). The EC50 values were also calculated using a sigmoid dose-response curve fit with variable slope in the program GraphPad Prism 4.0.

For dose-response curves for negative cooperativity (accelerated dissociation), 2.0 × 107 293EBNA IGF-IR cells/ml were incubated with 125I-labeled IGF-I (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 in the same concentration of Hepes binding buffer, and 25 μl of cells were transferred to tubes containing increasing concentrations of cold ligand in 1 ml of Hepes binding buffer. The dissociation of prebound ligand was stopped after 30 min at 15 °C by centrifugation at 4000 rpm for 5 min. The membrane pellet was counted in a gamma counter. The assays were performed at least three times for each analogue.
Kinase Receptor Activation Assay—The kinase receptor activation assays were performed as previously described (29). Briefly, P6 IGF-IR cells were grown overnight in 96-well plates in a concentration of $2.5 \times 10^5$ cells/well and serum-starved for 4 h in serum-free Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin. Cells were stimulated for 10 min with increasing concentrations of ligand at 37 °C, 5% CO2. The reaction was stopped by cell lysis at 4 °C using lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM EGTA, pH 7.5) with 100 mM NaF, 2 mM Na₃VO₄, and protease inhibitor mixture (1 μl/ml). IGF-IR was captured by transferring the cell lysate to Greiner Lumitrac 600 plates precoated with 24-31 antibody (35). Phosphorylated IGF-IRs were detected by adding europium-labeled antiphosphotyrosine antibody (PY20, PerkinElmer Life Sciences). Time-resolved fluorescence was measured as described above.

[^3H]Thymidine Incorporation Assay—DNA synthesis was quantified as[^3H]thymidine (Amersham Biosciences) incorporation into genomic DNA according to an optimized method by C. Bonnesen and M. Oleksiewicz3 (previously described in Ref. 36). Briefly, L6 rat muscle myoblasts were starved in medium containing 0.1% serum and stimulated for 19 h in medium containing 0.1% serum supplemented with increasing concentrations of ligand. The cells were pulsed with 0.125 μCi/well[^3H]thymidine for 2 h and harvested onto glass-fiber filters using a MICRO 96TM Skatron harvester (Molecular Devices). The filters were counted in a Wallac MicroBeta Counter (PerkinElmer Life Sciences).

RESULTS

Expression, Quantification, and Characterization of the IGF-I Analogues—All the IGF-I analogues were successfully expressed in an E. coli expression system. Eight IGF-I analogues were expressed, six single-substituted analogues (E9A IGF-I, D12A IGF-I, F16A IGF-I, D53A IGF-I, L54A IGF-I, and E58A IGF-I), one double-substituted analogue (E9A,D12A IGF-I), and finally one triple-substituted analogue (E9A,D12A,E58A IGF-I). All analogues were of the correct mass as measured by mass spectrometry (data not shown).

Alanine substitution generally has little effect on the overall structure of a molecule (37). To confirm this was the case with these new analogues, we performed far UV CD. Misfolding of IGF-I analogues can be clearly identified by a significant change in the far UV spectrum from the spectrum seen for IGF-I (38). All eight IGF-I analogues exhibited almost identical far UV CD spectra as seen for IGF-I (Fig. 3), confirming that the substituted alanines had little overall effect on secondary structure and that correct folding was achieved.

Binding Properties of the IGF-I Analogues—The affinities of the analogues were tested in two different competition assays using either a soluble receptor assay where the IGF-IR from lysed P6 IGF-IR cells are captured by 24-31 antibody or a whole cell receptor assay using 293EBNA IGF-IR cells.

The average EC₅₀ values obtained in the soluble receptor assay and the average EC₅₀ and $K_d$ values obtained for the whole cell assay for each analogue are shown in Table 1. The curves are illustrated in Fig. 4, A–C, for the whole cell receptor assay. Because the $K_d$ values and EC₅₀ values are comparable for the whole cell receptor assay, the following comparisons between the two assays are made using the EC₅₀ values.

IGF-I has almost identical binding affinities in both assays (0.29 versus 0.35 nM). F16A IGF-I (0.58 versus 1.04 nM), D53A IGF-I (0.40 versus 0.50 nM), and L54A IGF-I (1.20 versus 1.57 nM) also show similar affinities in the two types of assay (Table 1 and Fig. 4, A and B). However, three of the analogues clearly

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[^3]: C. Bonnesen and M. Oleksiewicz, manuscript in preparation.
TABLE 1

| Ligand              | Soluble IGF-IR | 293EBNA IGF-IR |
|---------------------|----------------|----------------|
|                     | EC50 ± S.D.    | Rel EC50       | Kd ± S.D. | Rel Kd |
| IGF-I               | 0.29 ± 0.03    | 100            | 0.35 ± 0.04 | 100 |
| E9A IGF-I           | 0.86 ± 0.20    | 34             | 3.87 ± 0.30 | 9     |
| D12A IGF-I          | 0.99 ± 0.28    | 29             | 1.89 ± 0.50 | 19    |
| F16A IGF-I          | 0.58 ± 0.02    | 50             | 1.04 ± 0.16 | 34    |
| D53A IGF-I          | 0.40 ± 0.04    | 73             | 0.50 ± 0.08 | 70    |
| L54A IGF-I          | 1.20 ± 0.09    | 24             | 1.57 ± 0.57 | 22    |
| E58A IGF-I          | 1.88 ± 0.60    | 15             | 4.50 ± 1.08 | 8     |
| E9A,D12A IGF-I      | >10            | <3             | >10        | >10   |
| E9A,D12A,E58A IGF-I| >100           | <1             | >100       | <1    |

FIGURE 4. Competitive binding of IGF-I and the IGF-I analogues to the IGF-IR. Binding curves showing the competing effect of IGF-I (●), E9A IGF-I (□), D12A IGF-I (○), and F16A IGF-I (●), L54A IGF-I (△), and E58A IGF-I (◇) for the IGF-IR. Increasing concentration of the ligands compete with a fixed concentration of 125I-labeled IGF-I for the IGF-IR using 293EBNA IGF-IR cells. Curves are shown as specific binding/specific binding at 0 μM cold ligand and are plotted using the average of three assays with each concentration measured in duplicate. S.D. are shown for all the data (smaller than the symbol when not visible).

have different affinities when analyzed in the two types of assays. E9A IGF-I has an EC50 value of 0.86 nM in the soluble receptor assay and 3.87 nM in the whole cell assay (Table 1, Fig. 4A). D12A IGF-I has an EC50 of 0.99 nM in the soluble receptor assay and 1.89 nM in the whole cell assay (Table 1, Fig. 4A). Finally, E58A IGF-I has an EC50 of 1.88 nM in the soluble receptor assay and 4.50 nM in the whole cell assay (Table 1, Fig. 4B).

In summary, substitution of residue 53 with alanine has almost no effect on binding, but in position 16 it results in a 2.0–2.9-fold change in affinity and, in position 54, a 4.2–4.5-fold reduction in affinity. Introducing alanine into position 9 leads to a 3.0–11.0-fold reduction, into position 12 leads to a 3.5–5.4-fold reduction, and finally into position 58 leads to a 6.6–12.8-fold reduction in affinity for the IGF-IR. Combining the E9A, D12A, and E58A substitutions into E9A,D12A IGF-I and E9A,D12A,E58A IGF-I resulted in two analogues with very poor binding affinities for the IGF-IR. The affinity was less than 3% that of IGF-I for E9A,D12A IGF-I and less than 1% of IGF-I for E9A,D12A,E58A IGF-I (Table 1 and Fig. 4C). Therefore, five of the six residues analyzed contribute to the binding energy. When combined into the double- and triple-substituted analogues, Glu9, Asp12, and Glu58 contribute in an additive manner.

To further analyze the binding properties of the six analogues, dose-response curves for negative cooperativity were performed using the 293EBNA IGF-IR cells. The potencies with which the single-substituted IGF-I analogues accelerated the dissociation of prebound labeled IGF-I correlated very well with the affinities seen in the whole cell competition binding assay (Fig. 5, A and B). However, E9A IGF-I was very poor at accelerating the dissociation even at very high concentrations of cold ligand compared with the other five analogues (Fig. 5A). The potency of the double-substituted IGF-I analogue looked like that seen for E9A IGF-I, whereas increasing concentrations of the triple-substituted IGF-I analogues resulted in no dissociation of prebound labeled IGF-I (Fig. 5C).

Receptor Phosphorylation Properties of the IGF-I Analogues—The ability to induce receptor phosphorylation by the eight analogues together with IGF-I was measured using the kinase receptor activation assay to determine whether changes in affinity correlated with changes in receptor phosphorylation. IGF-I stimulation for 10 min resulted in a dose response curve with maximum stimulation at 10 nM (Fig. 6). E9A IGF-I was poor at inducing receptor phosphorylation (Fig. 6A). It only reached 1/4 of the maximum seen for IGF-I. Nor was D12A IGF-I as potent as IGF-I at inducing IGF-IR phosphorylation (Fig. 6A). F16A IGF-I and D53A IGF-I showed similar phosphorylation patterns to IGF-I, which correlates well with the small changes in affinity seen for these analogues (Fig. 6B). L54A IGF-I and E58A IGF-I showed reduced potency in inducing receptor phosphorylation. However, both analogues reached the same maximum as seen for IGF-I. The reduced potency again correlated very well with the affinities seen for the two analogues (Fig. 6C). Finally, E9A,D12A IGF-I and
E9A,D12A IGF-I showed a clear reduction in ability to induce receptor phosphorylation (Fig. 6) both in terms of potency and maximal response.

**Mitogenic Potency of the IGF-I Analogues** — The biological outcome of the changes in affinity and receptor phosphorylation was evaluated by measuring [3H]thymidine incorporation in L6 rat muscle myoblasts treated with increasing concentrations of IGF-I (X), E9A IGF-I (■), D12A IGF-I (□), and F16A IGF-I (○) (A), IGF-I (×), D53A IGF-I (○), L54A IGF-I (▲), and E58A IGF-I (△) (B), and IGF-I (×), E9A,D12A IGF-I (▲) (C), and E9A,D12A,E58A IGF-I (△) (D). The P6 IGF-IR cell line overexpressing the IGF-IR was used. The results are illustrated as percentage of the IGF-I response at the concentration 10⁻⁸ M. Curves are shown as one representative assay of three assays with each concentration performed in triplicate. S.D. are shown for all the data (smaller than symbol when not visible).

E9A,D12A,E58A IGF-I showed a clear reduction in ability to induce receptor phosphorylation (Fig. 6D) both in terms of potency and maximal response.

E9A IGF-I showed a reduced potency for [3H]thymidine incorporation (Fig. 7A). However, cell growth was observed, and E9A IGF-I reached 80% of the response to IGF-I at the highest concentration (Fig. 7A). D12A IGF-I, which also showed reduced receptor phosphorylation, induced a clear mitogenic response, and the curve reached the same maximum as seen for IGF-I (Fig. 7A). The dose-response curves for F16A IGF-I and D53A IGF-I reached a similar maximum to that for IGF-I (Fig. 7B); however, the kinetics of DNA synthesis induction by F16A IGF-I was different than IGF-I. L54A IGF-I and E58A IGF-I (Fig. 7C) also showed a clear mitogenic effect. L54A IGF-I showed a similar curve as IGF-I, with a maximum slightly higher than IGF-I, and E58A IGF-I showed reduced potency; the maximum was probably still not reached at the highest concentration used in this assay. Finally, both E9A,D12A IGF-I and E9A,D12A,E58A IGF-I were poor at inducing DNA synthesis, although E9A,D12A IGF-I was slightly more potent than E9A,D12A,E58A IGF-I, reflecting their relative affinities for the IGF-IR (Fig. 7D).

In general, there was not as big a difference between the [3H]thymidine incorporation dose-response curves for of the analogues and IGF-I as would be expected based on the differences in IGF-IR binding affinities. A similar observation was previously reported for other IGF-I analogues (39). However, E9A IGF-I, D12A IGF-I, and E58A IGF-I showed reduced

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4 T. Trüb and P. De Meyts, unpublished data.
growth-promoting potency, correlating with the lower affinity of these three analogues.

DISCUSSION

Because the binding properties of insulin for its receptor have been studied more extensively than the binding of IGF-I to the IGF-IR, we have used the knowledge of structure-activity relationships existing for insulin binding to explore the role of specific residues in IGF-I binding to the IGF-IR.

As already described, there is evidence that insulin comprises two separate binding surfaces; site 1 and site 2. Data support that insulin upon binding cross-links two distinct binding sites (sites 1 and 2) on the α subunits of each IR half, thereby creating high affinity binding of one insulin molecule within the IR dimer. A variety of biochemical and mutagenesis data, supported by the recent structure of the IR extracellular domain, have converged to help identify the two IR binding sites. Site 1 comprises the N-terminal L1 receptor domain together with a short peptide fragment of the C-terminal FnIII-2 insert domain (of unknown structure), probably cooperating in trans between the two α subunits, whereas site 2 of the IR probably comprises residues located in FnIII-1 and FnIII-2 modules together with the C-terminal portion of L2 (1, 11, 30, 40–48).

In the case of the IGF-IR, it is believed that the receptor site 1 also comprises the N-terminal part of the L1 domain and the C-terminal FnIII-2 insert domain. A third part of site 1 (and unique for the IGF-IR) is considered to be composed of part of the cysteine-rich domain and is probably involved in binding the C-domain of IGF-I. Finally, the site 2 on the IGF-IR is thought to incorporate parts of the FnIII-1 domain as is seen with the insulin-IR interaction (1, 11, 12, 26, 30, 43, 45, 46, 49–51).

The IGF-I residues involved in IGF-IR binding have not been as extensively studied as those residues of insulin binding to the IR. However, a number of residues in IGF-I that are homologous to key residues of the insulin site 1 binding surface are critical for IGF-I binding to the IGF-IR. IGF-I residues important for binding include Ala5, Phe23, Tyr24, Tyr31, Arg36, Arg37, Val44, Met59, Tyr60, and Ala62 (20–25). Asp12 has been shown to be important for IGF-IR binding (28) and could be part of site 2 since the position of Asp12 is equivalent to Glu113 in insulin, a residue that is part of insulin site 2. To investigate whether IGF-I utilizes residues similar to insulin site 2, eight IGF-I analogues were expressed, and their binding properties for the IGF-IR were analyzed. As illustrated in Fig. 2, B and C, these residues are located very similarly in the crystal structures of insulin and IGF-I. In contrast, there are distinct differences in the region of IGF-I corresponding to the last five residues of the B-domain. These residues together with the unique C-domain compose a different binding epitope compared with insulin. This binding epitope uses the larger binding pocket of IGF-IR (Fig. 2A), created by the L1 domain and cysteine-rich domain. Therefore, the main differences between IGF-I and insulin seem to affect the site 1 binding more than a putative site 2 binding (52).

The binding affinity of the analogues was analyzed in two different competition assays; that is, a soluble receptor assay and a whole cell receptor assay. As illustrated in Table 1, IGF-I showed a similar affinity for the IGF-IR in the two types of assays. The soluble holoreceptor showed nanomolar affinity similar to the receptor in the membrane. In contrast, three of the single-substituted IGF-I analogues (E9A IGF-I, D12A IGF-I, and E58A IGF-I) showed a 1.5–4-fold difference in relative affinity, with the highest changes in relative affinity seen in the whole cell receptor assays.

Because the binding of IGF-I to the IGF-IR is a dynamic process that probably involves flexible regions necessary for the autophosphorylation and activation of receptor, these differences could be explained by the lack of flexibility when the receptor is captured in the soluble receptor assay compared with the whole cell receptor assay where the receptor is located in the membrane of the cell. Similar findings were obtained in the alanine scanning of insulin, where most residues from insulin site 2 did not show reduced receptor binding affinities using a soluble receptor assay, whereas decreased affinities were
found in whole cell assays for SerA12, LeuA13, GluA17, HisB10, and LeuB17. Only alanine substitution at GluB13 resulted in lower affinities in both types of assay (12, 37).

Combining the competition binding data, E9A IGF-I, D12A IGF-I, F16A IGF-I, L54A IGF-I, and E58A IGF-I all show a significant reduction in affinity when analyzing the binding affinity in whole cell receptor assays (≥2 fold change in affinity). Only D53A IGF-I showed no change in affinity. The reason for using a 2-fold 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 (49, 53). The most important of these five residues found to have reduced IGF-IR binding affinities were Glu9 (HisB10), Asp12 (GluB13), and Glu58 (GluA17). This is in contrast to the insulin site 2 interaction where GluB13, LeuB17, and LeuA13 (12) contribute most to IR binding.

Jansson et al. (27) have previously showed that F16A IGF-I showed almost no binding to the IGF-IR, which is in contrast to the data presented here. The F16A IGF-I CD spectrum presented in this study looks almost identical to the spectrum for IGF-I, whereas there was some difference between the F16A IGF-I and IGF-1 CD spectra reported by Jansson et al. (27). Because the Jansson F16A IGF-I showed similar spectral characteristics to misfolded IGF-I, it is possible that their preparation was not correctly folded, and this could explain the differences in activities seen between these two studies.

Dose-response curves for negative cooperativity assays showed that E9A IGF-I was very poor at inducing dissociation of prebound labeled IGF-I. The fact that Glu9 is important for the accelerated dissociation was further supported by combining the E9A substitution with D12A and E58A into E9A,D12A,E58A IGF-I, which resulted in an analogue that was not able to accelerate the dissociation of prebound labeled IGF-I. The receptor phosphorylation data showed a similar pattern. E9A IGF-I, D12A IGF-I, and E58A IGF-I showed a reduction in potency in IGF-IR phosphorylation, which correlated with the low receptor affinity of these three ligands. E9AIGF-I and D12A IGF-I also showed a reduction in the maximum response. This can be explained by the low affinity of the ligands. However, in the case where E58A IGF-I reaches the same maximum as IGF-I, even though it shows a clear reduction in affinity, the reduction in receptor phosphorylation maximum could also be explained by a change in the cross-linking process of the two receptor halves leading to a change in the phosphorylation pattern (e.g. reduced phosphorylation of receptors in general or phosphorylation of only some of the sites in the intracellular β-subunit). The double-substituted and triple-substituted IGF-I analogues showed reductions in both potency and maxima of receptor phosphorylation.

Finally, the eight IGF-I analogues showed a higher mitogenic potency in relation to IGF-I than expected when considering the relative binding affinities and potencies in phosphorylation experiments. Both the receptor binding assays and phosphorylation assays reflect a short-term response to ligand stimulation, whereas the mitogenic assays are conducted over 21 h, which could explain why these differences arise.

Having established that 5 of the 6 residues investigated are indeed important for IGF-IR binding, the question remains of whether any of these are important for the cross-linking of the receptor halves. In the model of IGF-IR (Fig. 2) the acidic side chain of Glu9 is solvent-exposed to a high degree and points away from the docked receptor half in this model. The neighboring aspartic acid in position 12 is also solvent-exposed. As mentioned above, Ala8 also seems to have a role in binding (22), and because it is located next to Glu9 (Fig. 2A) it could very well play a structural role keeping Glu9 in the right position to make the receptor contact rather than a direct role in binding, as alanine generally is not believed to make protein-protein contacts. Finally, the acidic side chain in position 58 is not as solvent-exposed as the Glu9 and Asp12. There could either be a structural change upon binding that would result in surface exposure of this residue (maybe together with the methionine in position 59 (22)), or these residues could have a more structural role keeping IGF-I in the right local conformation, as these residues are located in the second α-helix of the A-domain.

In conclusion, introducing three alanine substitutions into IGF-I (i.e. E9A, D12A, and E58A) results in an analogue with very poor affinity for the IGF-IR. This analogue is not able to accelerate the dissociation of prebound IGF-I, indicating that it cannot bind to the free binding site on the receptor. Finally, the analogue shows a poor receptor phosphorylation and reduced mitogenic potency. These observations together with the single-substitution data show that IGF-I partially uses residues equivalent to the residues of insulin site 2 to achieve high affinity binding to the IGF-IR. It suggests that Glu9, Asp12, Phe16, Leu54, and Glu58 could constitute a site 2 of IGF-I; moreover, it seems likely that the adjacent Ala8 and Met59 (Fig. 2A), whose substitutions have also been reported to impair binding, are also part of this binding site.

A note of caution is that there is much less information available for linking the two putative sites of IGF-I to their respective binding sites on the IGF-IR than is available for the insulin receptor (e.g. photoaffinity cross-linking data or binding of ligand mutants to minimized receptor constructs, as reviewed in De Meyts and Whittaker (Ref. 12)). Therefore, alternative explanations should be kept in mind, such as a cis interaction with two sites within the same subunit. The trans cross-linking model is, however, strongly supported by the elegant complementation experiment of Chakravarty et al. (54).

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Note Added in Proof—After submission of this paper, Glendorf et al. (55) published an elegant systematic amino acid scanning of residues in the insulin B-chain central helix showing major effects of substitutions at B12 and B16 (in our site 1) and B10 and B13 (in our site 2), confirming the importance of the B-chain central helix as a receptor binding motif as previously shown by photoaffinity cross-linking (56).
