An Insulin-like Growth Factor I/Insulin Hybrid Exhibiting High Potency for Interaction with the Type I Insulin-like Growth Factor and Insulin Receptors of Placental Plasma Membranes*

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We have prepared by semisynthetic methods a two-chain insulin/insulin-like growth factor I hybrid that contains a synthetic peptide related to residues 22-41 of insulin-like growth factor I linked via peptide bond to ArgBz2 of des-octapeptide-(B23-B30)-insulin and have applied the analog to the analysis of ligand interactions with the type I insulin-like growth factor and insulin receptors of placental plasma membranes. Relative potencies for the inhibition of 125I-labeled insulin-like growth factor I binding to type I insulin-like growth factor receptors were 1.0:0.20:0.003 for insulin-like growth factor I, the hybrid analog, and insulin, respectively. Corresponding relative potencies for the inhibition of 125I-labeled insulin binding to insulin receptors were 0.007:0.28:1 for the three respective peptides. Additional studies identified that the hybrid analog interacts with only one of two populations of insulin-like growth factor I binding sites on placental plasma membranes and permitted the analysis of insulin-like growth factor I interactions with the separate populations of binding sites. We conclude that (a) des octapeptide-(B23-B30)-insulin can serve well as a scaffold to support structural elements of insulin-like growth factor I and insulin necessary for high affinity binding to their receptors, (b) major aspects of structure relevant to the conferment of receptor binding affinity lie in the COOH-terminal region of the insulin B chain and in the COOH-terminal region of the insulin-like growth factor I B domain and in its C domain, and (c) the evolution of ligand-receptor specificity in these systems has relied as much on restricting interactions (through the selective introduction of negative structural elements) as it has on enhancing interactions (through the introduction of affinity conferring elements of structure).

Insulin and insulin-like growth factor I (IGF-I) represent homologous peptides that bind to different, but homologous,

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* The abbreviations used are: IGF-I, insulin-like growth factor I; t-Boc, tertiarly-butyloxycarbonyl; HPLC, high performance liquid chromatography.

plasma membrane receptors to initiate their relevant biological activities (1-4). The amino acid sequences and domain structures of insulin and IGF-I are compared in Fig. 1a. Whereas the insulin receptor system largely affects the metabolic status of appropriate target cells, and the IGF-I receptor system largely affects the mitogenic and growth responses of target cells (5-8), the two systems can be seen under certain circumstances to overlap in terms of their respective activities and functions (Refs. 9-11). Since the selectivities of the two systems for one or the other hormone are often 100-500-fold, however, important questions concern how ligand-receptor specificity and affinity are directed by ligand structure, and how ligand structure might be changed to achieve analogs with activities modified in experimentally or therapeutically useful ways. Importantly, studies based on the crystal structure of insulin and on computer-assisted modeling have led to the proposal that IGF-I and proinsulin exhibit very similar three-dimensional structures (12). In the latter peptide, excision of the C domain during precursor processing results in the formation of two-chain insulin, whereas in the former, the C domain is retained. IGF-I also contains an 8-residue extension on the A domain (a region called the D domain) which is not shared by proinsulin (1). A comparison of the sequences of the A and B domains of IGF-I and insulin establishes that 17 of 20 evolutionarily invariant residues of insulin are retained in IGF-I (the exceptions being TyrB26 → Gln46, GlnA46 → Glu46, and AspA42 → Ala42), that the 25 of 51 residues present in human insulin are present in human IGF-I, and that 32 of 51 residues found in insulin from various species are also found in human IGF-I. Importantly, the positions of cysteine residues (and presumably of disulfide bonds) are identical in the two peptides. No identities exist, however, in the C domains of proinsulin and IGF-I.

Studies of structure-function relationships as they relate to the IGF-I system have taken two courses. In the first, various two-chain hybrids containing the B domain of insulin or IGF-I and the A domain of one or the other of the same two peptides (sometimes modified to include the D domain) have been constructed (13-15). Related experiments have demonstrated that, whereas none of these peptides retains high potency, a few exhibit affinities for interaction with the IGF-I receptor somewhat higher than that exhibited by native insulin (13-15). In the second set of studies, expression of altered synthetic genes for IGF-I has yielded single-chain analogs with defined structural changes. These studies have demonstrated (a) the retention of high receptor binding potency in analogs in which major portions of the IGF-I A and B domains have been made more homologous to insulin (16, 17), (b) the relative lack of importance of the D domain in IGF-I receptor interactions (18), (c) the important contribu-
Affinity Labeling Studies—Placental membranes were incubated with radiolabeled IGF-I, with or without competing unlabeled peptides, as for receptor binding studies, centrifuged, and washed with the phosphate buffer described above lacking polyethylene glycol. The pellets were suspended in 0.45 ml of phosphate buffer containing 20 mM bis-N-hydroxyaspartimido sulfoxide, and the resulting suspensions were incubated for 15 min at 4 °C. Membrane pellets obtained by centrifugation were resuspended in 0.1 ml of 50 mM Tris, 2% (w/v) sodium dodecyl sulfate, 0.1 M dithiothreitol, pH 6.5, boiled for 10 min, and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26). Gels were fixed, stained, and dried using standard procedures, and were subjected to autoradiography at -70 °C by use of Kodak X-Omat film and DuPont-Cronex Lightning Plus enhancing screens, as described (97).

RESULTS AND DISCUSSION

Our approach to the design of the hybrid IGF-I/insulin analog identified in this report considered (a) our previously published observations on the insulin system that emphasize the importance of residues in the B chain distal to the insulin core in directing the high affinity interaction of ligand with receptor (22, 23, 28), and (b) the belief that potential flexibility in the IGF-I COOH-terminal B domain and the IGF-I C domain should be subject to mimicry by a two-chain analog that contains structural elements essential to ligand-receptor recognition. Although details of our semisynthetic methods (which require trypsin-catalyzed peptide bond formation) precluded the introduction of the native sequence of IGF-I-(22-41) into the core of insulin characterized by des-octapeptide-(B23-B30)-insulin, the semisynthesis was readily approached by replacing each of IGF-I residues Arg36 and Arg37 by Lys.

Affinity binding of the hybrid analog was then investigated (Table I, the analog inhibited by half the binding of the [125I]-labeled ligand at a concentration of 2.9 nM, a value only 5.8 times greater than that required by IGF for equivalent inhibition). Non-specific binding was defined as the amount of radioactivity remaining membrane associated in the presence of 200 nM of the hybrid analog, and the potential for des-octapeptide-(B23-B30)-insulin to serve as a covalent carrier for the IGF-I-related icosapeptide.

Initial studies based on the two-chain IGF-I/insulin hybrid identified above examined the ability of the analog to compete for radiolabeled IGF-I binding to receptors present on isolated placental plasma membranes. As shown in Fig. 2a and in Table I, the analog inhibited by half the binding of the [125I]-labeled ligand at a concentration of 2.9 nM, a value only 5.8 times greater than that required by IGF for equivalent inhibition, notwithstanding that a full two-thirds of the peptide retains the structure of insulin. Importantly, the ability of the analog to compete for [125I]-labeled IGF-I binding to membrane receptors is 70 times greater than the corresponding ability of insulin. Also as shown in Fig. 2a and Table I, des-octapeptide-(B23-B30)-insulin (the scaffold for the IGF-I-related peptide to which it is linked in the IGF-I/insulin hybrid) has only about 0.004% of the apparent affinity of the hybrid analog for plasma membrane IGF-I receptors, whereas the IGF-I-based icosapeptide itself exhibits no detectable ability to compete for IGF-I binding. Even in an admixture of des-octapeptide-(B23-B30)-insulin and the icosapeptide, binding inhibition was no greater than that achieved by the des-octapeptide-insulin alone (data not shown). From the data presented above, it is clear that the two-chain IGF-I/insulin hybrid exhibits high affinity for the IGF-I receptor, whereas neither of its component parts (des-octapeptide-(B23-B30)-insulin or the IGF-I-related icosapeptide) is capable of inter-
An Insulin/Insulin-like Growth Factor I Hybrid

**FIG. 1. Structural relationships in the IGF-I/insulin hybrid.**
a illustrates the amino acid sequence of insulin, IGF-I, and the IGF-I/insulin hybrid by use of the one-letter code and the domain structures of the three peptides. The order of the domain structures in IGF-I is given by: B, C, A, D. Hyphens are used to indicate the extension of sequences from one domain to another. b illustrates the diagramatic structure of the hybrid and shows by vertical lines the placement of residues in the analog that occur within the sequence of IGF-I. The insulin A chain is shown as a light line, the insulin B chain-(l-22) as a heavy line, the IGF-related icosapeptide as a dashed line, and disulfide bonds as dotted lines. The amino termini of the two peptide chains are identified by large dots. c illustrates the potential three-dimensional structure of the hybrid. The structure of the insulin A chain and the insulin B chain-(1-22) (together comprising des-octapeptide-(B23-B30)-insulin) is taken from the crystallographic structure of insulin within the P-zinc hexamer (37). The sequence of the synthetic IGF-I-related icosapeptide which is attached to A$** of des-octapeptide-(B23-B30)-insulin by trypsin-catalyzed semisynthesis is shown explicitly by use of the one-letter amino acid code. The conformation of the icosapeptide-derived portion of the IGF-I/insulin hybrid is not known.

acting with the type I IGF receptor in an effective way. As important, this analog with high affinity for the IGF-I receptor inhibited at maximum only about 80% of the binding of the radiolabeled tracer. Thus, as shown in Fig. 2a, and as identified before for insulin and for other much lower affinity insulin analogs (21), use of the two-chain IGF-I/insulin hybrid results in the identification of heterogeneity in placental plasma membrane binding sites for IGF-I. The nonparallel character of the binding competition curves of Fig. 2a implies that ligand association with the type I IGF receptor is subject to kinetic complexity which would be consistent, in some cases, with negative homotropic interactions.

Additional experiments addressed the affinity of the hybrid analog for the insulin receptor of placental membranes. As shown in Fig. 2b and Table I, the hybrid analog inhibited by half the binding of 125I-labeled insulin to plasma membrane insulin receptors at a concentration of 2.8 nM, a value only X&fold higher than the concentration of insulin required to accomplish the equivalent inhibition, and a value (both relatively and absolutely) close to the concentration of hormone

**FIG. 2. Inhibition of ^125^I-labeled IGF-I and ^125^I-labeled insulin binding to isolated human placental membranes by the corresponding unlabeled hormones, the hybrid IGF-I/insulin analog, and additional peptides.** a, inhibition of binding of ^125^I-labeled IGF-I; b, inhibition of binding of ^125^I-labeled insulin. Both panels plot the amount of radiolabeled hormone which became membrane-associated in the presence of competing peptides divided by the amount of radiolabeled peptide which became membrane associated in the absence of competitor (B/Beq) versus the concentration of inhibitory peptide on a logarithmic scale. The identifying numbers provided in Table I (rather than symbols) are used to indicate individual peptides: 1, IGF-I; 2, insulin; 3, the hybrid IGF-I/insulin analog; 4, des-octapeptide-(B23-B30)-insulin; 5, the IGF-I-related icosapeptide; 6, human insulin-(B23-B30) (the COOH-terminal octapeptide of the insulin B chain). Note that the ordinate scale in panel b only has been extended below zero for clarity of presentation. See "Experimental Procedures" and the text for further details.
causing half-maximal inhibition of IGF-I binding to type I IGF receptors in the same membrane preparation. IGF-I itself showed only low affinity for the insulin receptor of placental plasma membranes (the concentration of the growth factor causing half-maximal inhibition of $^{125}$I-labeled insulin binding being about 150-fold greater than the required concentration of insulin) and des-octapeptide-(B23–B30)-insulin exhibited only about 1/1000 of the affinity of insulin for the insulin receptor (Fig. 2a and Table 1). Interestingly, the IGF-I-related octapeptide exhibited an extremely low, but detectable, potency in inhibiting insulin binding to plasma membrane insulin receptors, whereas the native octapeptide corresponding to residues 23–30 of the insulin B chain was without effect. As is clear from Fig. 2b, both insulin and the hybrid analog inhibited $^{125}$I-labeled insulin binding to plasma membrane receptors completely and in an apparently homogeneous way.

Since the inhibition of $^{125}$I-labeled IGF-I binding to placental plasma membranes by the IGF-I/insulin hybrid was shown above to be incomplete, even at high analog concentrations (Fig. 2a), and since the hybrid analog retains (unlike insulin itself) a high affinity for the major fraction of IGF-I binding sites on placental plasma membranes, we were encouraged to use the analog as a probe for IGF-I receptor site heterogeneity. Studies were therefore undertaken in which IGF-I was allowed to compete for radiolabeled ligand binding in the presence of $1 \mu M$ analog or $100 \mu M$ insulin (concentrations of these peptides that cause maximal effect, cf. Fig. 2a). The inset to Fig. 3 demonstrates that the concentration of IGF-I causing half-maximal inhibition of radiolabeled ligand binding to type I IGF receptors on placental membranes is in the range 0.6–3.0 nM whether one considers total binding observed in the absence of additional peptides or the approximately 20% of total binding that remains uninhibited in the presence of $1 \mu M$ IGF-I/insulin hybrid or $100 \mu M$ insulin. Analysis of related data by mathematical modeling (by use of computer-assisted, nonlinear, least squares methods and an expression for a single binding equilibrium (29)) identified that 26% of total IGF-I binding sites on placental plasma membranes arise from the fraction which is not sensitive to inhibition by high concentrations of insulin. It would thus appear that use of the high affinity hybrid analog has identified two populations of IGF-I binding sites on placental membranes: both exhibit apparent dissociation constants for IGF-I $\approx 1$ nM, whereas only one (the major fraction) exhibits a very significant affinity for the IGF-I/insulin hybrid.

Importantly, the inset to Fig. 3 shows that $^{125}$I-labeled IGF-I which remains membrane bound even in the presence of 1.0

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**TABLE 1**

<table>
<thead>
<tr>
<th>Identifying number</th>
<th>Peptide</th>
<th>Concentration causing half-maximal inhibition of radiolabeled IGF-I binding</th>
<th>Concentration causing half-maximal inhibition of radiolabeled insulin binding</th>
<th>Potency relative to IGF-I binding sites</th>
<th>Potency relative to insulin receptor binding sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IGF-I</td>
<td>$0.5 \pm 0.2$</td>
<td>$130 \pm 29$</td>
<td>100</td>
<td>$0.7 \pm 3$</td>
</tr>
<tr>
<td>2</td>
<td>Insulin</td>
<td>$200 \pm 40$</td>
<td>$0.8 \pm 0.2$</td>
<td>$0.25 \pm 0.07$</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>IGF-I/insulin hybrid</td>
<td>$2.9 \pm 1.5$</td>
<td>$2.8 \pm 1.0$</td>
<td>$10 \pm 7$</td>
<td>$28 \pm 3$</td>
</tr>
<tr>
<td>4</td>
<td>Des-octapeptide-(B23–B30)-insulin</td>
<td>$&gt;1,000,000$</td>
<td>$870 \pm 150$</td>
<td>$&lt;0.000006$</td>
<td>$0.09 \pm 0.04$</td>
</tr>
<tr>
<td>5</td>
<td>IGF-I-related octapeptide</td>
<td>$&gt;1,000,000$</td>
<td>$23,000 \pm 11,000$</td>
<td>$&lt;0.00006$</td>
<td>$0.003 \pm 0$</td>
</tr>
<tr>
<td>6</td>
<td>Insulin-(B23–B30)</td>
<td>$&gt;1,000,000$</td>
<td>$&gt;1,000,000$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 3.** Inhibition of $^{125}$I-labeled IGF-I binding to isolated human placental membranes by IGF-I in the presence or absence of additional peptides. A, inhibition by IGF-I without further additions; B, inhibition in the presence of $1 \mu M$ IGF-I/insulin hybrid; C, inhibition in the presence of $100 \mu M$ insulin. Data have been normalized to reflect the varying amounts of radioactivity that became membrane-associated in the absence of unlabeled IGF-I for the separate conditions under which no other peptide is added or under which high concentrations of the IGF-I/insulin hybrid or insulin are present (about 20% of the control value, cf. Fig. 2a). The inset shows an autoradiogram resulting from the polyacrylamide gel electrophoresis of solubilized membranes which had been incubated with $^{125}$I-labeled IGF-I under various conditions and which had then been subjected to chemical cross-linking. Lane A, membranes incubated with radiolabeled IGF-I alone (material representing about one-fifth of a typical incubation to account for the decreased binding of IGF-I observed in the presence of high concentrations of the IGF-I/insulin hybrid or of insulin, see above); lane B, membranes incubated with radiolabeled IGF-I in the presence of $0.1 \mu M$ IGF-I; lane C, membranes incubated with radiolabeled IGF-I in the presence of $1 \mu M$ IGF-I/insulin hybrid; lane D, membranes incubated with radiolabeled IGF-I in the presence of $100 \mu M$ insulin. See "Experimental Procedures," the legend to Fig. 2, and the text for further details.

$\mu M$ IGF-I/insulin hybrid or $100 \mu M$ insulin is associated primarily with a protein having an apparent molecular weight of 130,000, the molecular weight of the ligand-binding $\alpha$-subunit of the type I IGF receptor (2). Although the lower bands in the inset to Fig. 3 may correspond to $^{125}$I-labeled IGF-I cross-linked to IGF-binding proteins (cf. Ref. 30), the absence of a band corresponding to a molecular weight 260,000 protein excludes the possibility that $^{125}$I-labeled IGF-I remaining membrane-associated in the presence of high concentrations of insulin or the hybrid is bound to the type II IGF receptor (cf. Ref. 31). Taken together, the results of Fig. 3 identify the existence of two populations of specific type I IGF receptors on placental plasma membranes. The extent to which the proportions of the two binding sites might vary...
among different cell types, and the potentially separate linkages of each site to different cellular responses, of course, remains to be investigated, as do the relationships of this IGF-I binding site heterogeneity to IGF-I binding site heterogeneity detected previously by reference to the use of antibodies to the type I IGF receptor (32, 33) or to the degree of oligomerization of the type I IGF receptor heterodimer (34, 35).

Whereas the data of Fig. 2, a and b, and Table I identify that the hybrid IGF-I/insulin analog competes for radiolabeled IGF-I receptors about 25% as well as IGF-I, and competes for radiolabeled insulin binding to insulin receptors about 30% as well as insulin (thereby documenting the nearly equivalent potencies of the analog for the type I IGF receptors and insulin receptors of the placental plasma membrane), they also identify several important relationships between the IGF-I and insulin systems.

First, notwithstanding that the IGF-I system exhibits about a 420-fold selectivity for IGF-I relative to insulin, and that the insulin system exhibits about a 170-fold selectivity for insulin relative to IGF-I, both systems exhibit nearly equivalent selectivity (or lack of it) for the IGF-I/insulin hybrid. Thus, des-octapeptide-(B23–B30)-insulin appears to serve as a scaffold for the IGF-I-related icosapeptide in inducing ligand interactions with both the major fraction of IGF-I binding sites and the insulin binding sites of placental plasma membranes. The failure of the hybrid to interact with all IGF-I binding sites on placental membranes suggests, however, that structural elements in the insulin-derived scaffold (rather than the lack of importance of the COOH-terminal B domain) may limit the interaction of the analog with the minor binding site population.

Second, linkage of the COOH-terminal insulin B chain octapeptide to the des-octapeptide-(B23–B30)-insulin scaffold induces a 1000-fold, or greater, increase in the apparent affinity of receptors for interaction with the peptide, whether the receptor system under study is that of IGF-I or insulin. Nevertheless, whereas the 1000-fold increase in affinity attending linkage of des-octapeptide-(B23–B30)-insulin to the insulin octapeptide is sufficient to induce a subnanomolar dissociation constant for the insulin receptor, the related linkage falls short (by about 400-fold) in inducing high affinity interaction with the type I IGF receptor. The details of relevant structure for high affinity interaction with the IGF-I receptor thus extend beyond what can be provided by insulin itself.

Third, since neither des-octapeptide-(B23–B30) insulin nor the IGF-I-related icosapeptide can account (alone or in admixture) for the high affinity of the IGF-I/insulin hybrid for the IGF-I or the insulin receptor systems, it would appear that the two domains of the hybrid analog behave cooperatively in inducing a high affinity state of ligand-receptor interaction with the two receptors. The IGF-I system apparently exhibits the greater degree of cooperativity in that linkage of the synthetic icosapeptide to des-octapeptide-(B23–B30)-insulin induces at least a 300,000-fold increase in the affinity of the latter peptide for the IGF-I receptor, whereas it induces about a 300-fold increase in the affinity of the truncated insulin for the insulin receptor.

Fourth, the presence of the IGF-I-related icosapeptide on the scaffold provided by des-octapeptide-(B23–B30)-insulin inhibits only slightly the interaction of ligand with the insulin receptor. This result is consistent with the general need for a residue with a β-aromatic ring at position B25 for high affinity insulin-receptor interactions (22, 23), with the relative lack of importance of the detailed structure of the COOH-terminal B chain domain distal to residue B25 in those interactions (22), and with the limited, but clear acceptance of COOH-terminal B-chain extensions in the interactions of des-Lys10-Arg15-proinsulin with the insulin receptor (36).

Fifth, notwithstanding the fact that the IGF-I/insulin hybrid retains a two-chain structure, its affinity for IGF-I receptor interactions exceeds that of other two-chain hybrids synthesized to date by many fold. Since related experiments have shown that α-Gly5, α-Phe8, Lys20-tris-acetyl-insulin and human proinsulin actually exhibit lower binding potency for the type I IGF receptor than that exhibited by insulin (21), there is little cause to imagine that the presence of a free A domain α-amino group, or the two-chain nature of the structure in the IGF-I/insulin hybrid, contribute importantly to its somewhat lower than normal affinity for the IGF-I receptor of placental plasma membranes.

Given the ease with which a ligand with nearly equivalent affinity for insulin and type I IGF receptors can be constructed, and the separate roles that these two peptides play in animal physiology, it is important to question not only how affinity of the two ligands for their respective receptors has been enhanced during evolution, but also how selectivity has been introduced. On the one hand, it appears that the COOH-terminal B chain domain of insulin and the B chain plus C peptide domains of proinsulin fail to provide elements of structure critical to the potential interactions of these peptides with the IGF-I receptor. On the other hand, it appears that replacements which have occurred in the IGF-I/A domain and in the NH2-terminal IGF-I B domain (both relative to insulin) play an active role in proscribing the high affinity interaction of IGF-I with the insulin receptor. It thus seems that the insulin scaffold would actually serve well for conferring affinity in both receptor systems, but that the scaffold has been adjusted during evolution to ensure that structural elements which provide binding specificity are developed quite separately from those which provide direct binding energy. Application of these ideas to the insulin and insulin-like growth factor systems by use of analogs prepared by semisynthetic or other methods should help to clarify how these systems developed their selectivities and how their characteristics can be modified by selective structural change.

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An Insulin/Insulin-like Growth Factor I Hybrid


An insulin-like growth factor I/insulin hybrid exhibiting high potency for interaction with the type I insulin-like growth factor and insulin receptors of placental plasma membranes.
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