An atypical insulin receptor with high affinity for insulin-like growth factors copurified with placental insulin receptors

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Communicated by G. J. V. Nossal, January 27, 1986

ABSTRACT Insulin receptors purified from human placenta by sequential affinity chromatography on wheat germ lectin-agarose and insulin coupled to 1,1'-carbonyldimidazole-activated agarose (CDI-agarose) retained full binding activity but bound a greater than predicted amount of 125I-labeled insulin-like growth factor I (IGF-I). IGF-I and multiplication-stimulating activity (MSA; the rat homologue of IGF-II) were equipotent in displacing either 125I-labeled IGF-I or 125I-labeled MSA from the purified receptors; insulin was 5-15 times more potent. Competitive binding studies indicated that this IGF binding activity could not be explained by cross-reactivity with classical insulin receptors or by coelution of IGF-I or IGF-II receptors. Instead, it was due to a minor population of discrete atypical insulin receptors (6-18%) total insulin receptors with moderately high affinity (Kd = 2-4 x 10^-9 M) for IGF-I and MSA. These receptors were not an artifact of insulin-CDI-agarose chromatography, since they were present in wheat germ lectin-agarose-purified preparations and could also be purified from insulin-succinylidiaminodipropylamino-agarose. Affinity labeling with 125I-labeled MSA revealed that these atypical receptors had the same binding subunit (Mr, 140,000) as classical insulin and IGF-I receptors. They displayed intermediate reactivity with polyclonal and monoclonal antibodies to the insulin and IGF-I receptors. It is therefore likely that insulin receptors purified by immunoadsorption would also contain atypical insulin receptors. The finding of more than one type of insulin receptor might relate to the slight variations in the cDNA nucleotide sequences and the multiple mRNA species reported for the insulin receptor (Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graff, L., Clausner, E., Ou, J.-H., Masiarz, F., Kau, Y. W., Goldfine, I. D., Roth, R. A. & Rutter, W. J. (1985) Cell 40, 747-758).

Insulin receptors have been purified to homogeneity from human placental membranes by sequential affinity chromatography on wheat germ lectin-agarose and insulin covalently linked to succinylidiaminodipropylamino-agarose (1). Using the same conditions of elution, but using an affinity resin consisting of insulin coupled to 1,1'-carbonyldimidazole-activated agarose (CDI-agarose), workers in our laboratory have also purified placental insulin receptors with full binding activity (6 nmol of insulin per mg of protein, equivalent to 2 mol of insulin per mol of receptor) comprised of subunits of Mr, 130,000 (α) and Mr, 90,000 (β) (2). However, since receptors for insulin-like growth factor I (IGF-I) in human placenta bind to wheat germ lectin-agarose (3), crossreact with insulin with 1/200th the affinity of insulin for its own receptor (4), and have a similar subunit composition to the insulin receptor (3, 5-7), we questioned whether these receptors might also be affinity-purified with insulin receptors. Initially, we found that receptors purified from insulin-CDI-agarose bound 125I-labeled IGF-I to a greater extent than predicted from the known crossreactivity of IGF-I with insulin receptors (8). In the present report, this IGF-I binding activity is characterized and shown to be due to a distinct species of insulin receptor.

MATERIALS AND METHODS

Materials. Porcine monocomponent insulin (Actrapid solution, 100 units/ml) was purchased from Novo (Copenhagen, Denmark). IGF-I was either purchased from Amgen (Thousand Oaks, CA), or was kindly provided by R. Himmel, Zurich (preparation 1/4). Multiplication-stimulating activity (MSA; the rat homologue of IGF-II) was purified from the conditioned medium of BRL-3A rat liver cells as described (9). Cohn fraction IV of human plasma (Commonwealth Serum Laboratories, Melbourne), acidified and subjected to cation exchange chromatography on SP Sephadex C-25 (10), was used as a source of IGFs for estimation of nonspecific binding. One microliter (≈0.05% pure) contained the equivalent of 80 ng of IGF-I and 0.8 ng of insulin (determined by immunoassay). Insulin, Amgen IGF-I, and MSA were labeled with 125I by the fractional chloramine-T procedure (11) to specific activities of 100-130 μCi/μg, 320-430 μCi/μg, and 130-200 μCi/μg, respectively (1 Ci = 37 GBq), and purified by chromatography on cellulose CFII (11). For some experiments (see legends to Figs. 2 and 5), 125I-labeled IGF-I was purified by hydrophobic interaction chromatography (12) on octyl-Sepharose CL-4B (Pharmacia).

Human immunoglobulin (Cohn fraction II) and Staphylococcus aureus [strain Cowan I, supplied as a formalin-fixed heat-killed 10% (wt/vol) suspension] were purchased from the Commonwealth Serum Laboratories (Melbourne); Triton X-100 and bovine serum albumin (RIA grade) were from Sigma; disuccinimidyl suberate and 1,1'-carbonyldimidazole (CDI-activated agarose [Reacti-Gel (6x)]) were from Pierce; bovine serum albumin (Pentex) was from Miles; cellulose powder CFII was from Whatman; and polyethylene glycol 6000 (PEG) was from BDH. All reagents for NaDodSO4/ PAGE were from Bio-Rad Laboratories.

Antisera containing anti-receptor antibodies were from patients B-2 and B-10 with the type B syndrome of insulin resistance and acanthosis nigricans (13, 14). Immunoglobulin G (IgG) was prepared from serum B-10 by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia). Monoclonal antibodies to the insulin receptor (αIR-1) and to the IGF-I receptor (αIR-3) (7) were kindly donated by S. Jacobs (Wellcome). Rabbit antiserum to mouse immunoglobulins was purchased from Dakopatts (Denmark).

Insulin-CDI-agarose was prepared as described (2). Insulin receptors with full binding activity were purified to homo-

Abbreviations: CDI-agarose, 1,1'-carbonyldimidazole-activated agarose; IGF-I, insulin-like growth factor I; MSA, multiplication-stimulating activity.
geneity from human placenta by sequential affinity chromatography on wheat germ lectin-agarose and insulin-CDI-agarose (2). In one experiment, insulin affinity chromatography was performed on a column of insulin-succinyl/diaminodipropylamino-agarose prepared by D. Hobson by the procedures outlined in ref. 1. Wheat germ lectin-agarose-purified glycoproteins were adsorbed, washed, and eluted from this column in the same fashion as described in ref. 2.

**Binding Assays.** Eluates from the wheat germ lectin-agarose column (20–80 µl; 12–80 µg of protein) or the insulin-CDI-agarose column (20–90 µl; 25–240 ng of protein) were incubated with 10,000–15,000 cpm of 125I-labeled insulin, 125I-labeled IGF-I, or 125I-labeled MSA for 18–24 hr at 4°C in a total volume of 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing Triton X-100 (1.0 g per 100 ml). In competition binding studies, when unlabeled insulin, IGF-I (preparation I/4) or MSA was included, incubation mixtures also contained 0.25 g per 100 ml of bovine serum albumin. Receptor-bound hormone was precipitated by PEG (final concentration, 12.5 g per 100 ml) in the presence of carrier human immunoglobulin (final concentration, 0.05 g per 100 ml).

To assess specific labeled insulin, 125I-labeled IGF-I, or 125I-labeled MSA to their receptors, the nonspecific binding of radioactivity in the presence of unlabeled insulin (5 µg) or unlabeled IGF-I (1 µl of 0.05% pure preparation) was subtracted from total binding. Data from the competition binding studies were analyzed by the method of Scatchard (15).

**Affinity Labeling of Purified Receptors.** Purified receptors at final protein concentrations of 300 and 600 ng/ml were incubated at 4°C for 20 hr with 125I-labeled insulin (25 ng/ml) or 125I-labeled MSA (5 µg/ml), respectively, in the presence or absence of excess unlabeled hormones in 0.1 M sodium phosphate buffer containing Triton X-100 (0.1 g per 100 ml) and bovine serum albumin (1.0 g per 100 ml). Disuccinimidyl suberate, freshly dissolved in dimethyl sulfoxide (100 mM) was added to a final concentration of 1 mM. After 15 min at 0°C, 50-µl aliquots were boiled for 5 min with equal volumes of twice concentrated NaDodSO4/PAGE sample buffer containing 0.1 M dithiothreitol, and subjected to NaDodSO4/PAGE and autoradiography as reported (16).

**RESULTS**

**Binding Studies with Affinity-Purified Receptors.** Elution profiles from the insulin-CDI-agarose column for 125I-labeled IGF-I and 125I-labeled insulin binding activities were identical. Specific binding at 125I-labeled IGF-I and 125I-labeled insulin, expressed as bound/free per 100 ng of protein, ranged from 0.07 to 0.26 and from 0.50 to 1.02, respectively.

In the same preparations of purified receptor, the specific binding of 125I-labeled IGF-I was 4th–5th that for 125I-labeled insulin—i.e., 7–25 times higher than predicted from 1% crossreactivity with the insulin receptor.

To resolve the identity of the IGF-I binding activity copurifying with insulin receptors, competition binding studies were performed with insulin, IGF-I, and MSA (Fig. 1). IGF-I and MSA were roughly equipotent in displacing tracer 125I-labeled IGF-I or 125I-labeled MSA from their binding sites. Both hormones bound with moderate affinity to these receptors: concentrations of 15–50 ng/ml were required to displace 50% of specific tracer binding and the dissociation constants for IGF-I binding ranged from 2–4 × 10⁻⁷ M (Fig. 2). On the other hand, far lower concentrations of insulin (2–5 ng/ml) were required to displace 50% of specifically bound 125I-labeled IGF-I or 125I-labeled MSA. These findings indicate that the IGF binding activity copurifying with insulin receptors was due to an atypical insulin receptor with moderate affinities for the insulin-like growth factors, IGF-I and MSA. It was not due to copurification of IGF-I or IGF-II receptors, because IGF-I receptors have a characteristic pattern of crossreactivity [IGF-I > MSA > insulin (6, 17); Fig. 3] and IGF-II receptors are insulin insensitive (6, 17). IGF-I receptors are responsible for most of the IGF-I binding activity in solubilized human placental membranes (Figs. 2 and 3) and do not bind to insulin-CDI-agarose (Fig. 3).

Consistent with the idea that 125I-labeled IGF-I was not crossreacting to an appreciable extent with the classical insulin receptors from the affinity column, the relative potencies of unlabeled insulin, MSA, and IGF-I for displacing 125I-labeled insulin from the purified receptors were quite different from their relative potencies for displacing 125I-labeled IGF-I (Fig. 4). Insulin, as expected (4, 7), was 85–145 times more potent than IGF-I. Moreover, Scatchard analyses of IGF-I and insulin binding in four different preparations of purified receptor revealed that the number of IGF-I binding
Immunological Properties of the Atypical Insulin Receptor: Comparison with Classical Insulin and IGF-I Receptors. Receptors from the insulin affinity column, labeled with \(^{125}\)I-labeled insulin (>90% classical insulin receptors), were precipitated completely by antisera B-2 and B-10, while classical IGF-I receptors not bound to the column, labeled with \(^{125}\)I-labeled IGF-I, were only 25% precipitated by antiserum B-2 and not at all by antiserum B-10. Intermediate reactivity was shown by the \(^{125}\)I-labeled IGF-I-labeled sites eluting from the column (atypical insulin receptors): 46% precipitation with antiserum B-2 and 22% with antiserum B-10 (Fig. 5).

The reactivity of the monoclonal antibodies with the classical insulin and IGF-I receptors was predictable (7, 18): IR-1 precipitated 45% of affinity-purified \(^{125}\)I-labeled insulin-labeled receptors and an insignificant proportion (<3%) of the classical IGF-I receptors in the unbound fraction. aIR-3 inhibited \(^{125}\)I-labeled IGF-I binding to classical IGF-I receptors by 78% and had no effect on \(^{125}\)I-labeled insulin binding.

With the atypical receptors, aIRI-1 precipitated only 14% of the bound \(^{125}\)I-labeled IGF-I and IR-3 inhibited IGF-I binding by 50%.

Storage Stability of Affinity-Purified Receptors. The \(^{125}\)I-labeled IGF-I and \(^{125}\)I-labeled insulin binding activities of purified receptors remained constant during storage at 4°C for

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**Fig. 3.** Competition between \(^{125}\)I-labeled IGF-I and increasing concentrations of unlabeled IGF-I ( ), MSA ( ), or insulin ( ) for binding to receptors eluted from wheat germ lectin-agarose (Upper) or remaining in the flow-through of the insulin-CDI-agarose column (Lower). Assay tubes contained 50 µl of wheat germ lectin-agarose eluate (36 µg) or 45 µl of insulin-CDI-agarose flow-through (32 µg). Sites was only 11% ± 5% (mean ± SD) of the total insulin binding sites (Fig. 2).

**Fig. 4.** Competition between \(^{125}\)I-labeled insulin and increasing concentrations of unlabeled insulin ( ), MSA ( ), or IGF-I ( ) for binding to receptors in wheat germ lectin-agarose eluates before (Upper) and after (Lower) elution from insulin-CDI-agarose. Assay tubes contained 50 µl of the same preparation of wheat germ eluate used in Fig. 3, or 30 µl of pooled neutralized insulin-CDI-agarose eluate. The latter (10 µl, 1.5 µg/ml) was derived from 8 ml of wheat germ lectin-agarose eluate (0.7 mg/ml). The relative potencies of the three unlabeled peptides in competing for specifically bound \(^{125}\)I-labeled insulin were the same regardless of whether the receptors were eluted from wheat germ lectin-agarose or insulin-CDI-agarose.

**Fig. 5.** Immunoprecipitation of \(^{125}\)I-labeled insulin-labeled ( ) or \(^{125}\)I-labeled IGF-I-labeled ( ) receptors purified from insulin-CDI-agarose or of \(^{125}\)I-labeled IGF-I-labeled receptors remaining unbound to the insulin-CDI-agarose column ( ) by antiserum B-2 (Upper) and antiserum B-10 IgG (Lower). Tracer amounts of labeled hormones were incubated with receptor preparations (25 ng or 12 µg of protein) for 20 hr at 4°C, then for another 24 hr with antiserum B-2 or antiserum B-10 IgG at the final dilutions/concentrations indicated. Antibody-bound receptors were precipitated by S. aureus (0.05 ml of 10% (wt/vol) suspension) and total receptors (antibody-bound and nonantibody-bound) by PEG. Specific PEG-precipitable binding (in the absence of antibodies) for the three hormone–receptor combinations was 41% ( ), 16% ( ), and 32% ( ). To assess specific binding of \(^{125}\)I-labeled insulin or \(^{125}\)I-labeled IGF-I to antibody-bound receptors, the nonspecific binding of radioactivity in the presence of control serum was subtracted from total binding. Specific immunoprecipitable tracer binding was expressed as a percentage of the specific PEG-precipitable binding.
13 days. However, differential effects were observed after storage at −20°C; 125I-labeled IGF-I binding activity remained stable, whereas 125I-labeled insulin binding activity declined rapidly with a half-life of 6 days. These results reinforce the conclusion that the IGF-I binding activity is not due to crossreactivity with classical insulin receptors or to a degradation product of these receptors, but rather to the presence of a discrete subpopulation of atypical receptors.

**Covalent Labeling and Structure of Atypical Insulin Receptors.** To define the structure of the IGF binding sites copurified on the insulin affinity column, covalent labeling with 125I-labeled MSA was performed by using the bifunctional chemical crosslinker disuccinimidyl suberate, in the presence and absence of low concentrations (25 ng/ml) of unlabeled insulin, MSA, and IGF-I. 125I-labeled MSA was used in preference to 125I-labeled IGF-I because, although both hormones bound with approximately equal affinities to the atypical insulin receptors (Fig. 1), 125I-labeled insulin would be more likely to detect IGF-II receptors, if present (6, 17). When subjected to NaDodSO4/PAGE under reducing conditions, chemically crosslinked 125I-labeled MSA appeared as a M₁, 140,000 complex, as did crosslinked 125I-labeled insulin (Fig. 1). Under nonreducing conditions, both labeled hormones migrated as M₂, 280,000, 300,000, and 330,000 complexes (data not shown). The autoradiographic intensity of the bands was significantly diminished by unlabeled insulin, and to a lesser extent by IGF-I and MSA (Fig. 6). In contrast, classic IGF-II receptors, after covalent labeling with 125I-labeled MSA, are resolved as M₂, 260,000 complexes in reduced gels and as M₂, 220,000 complexes in nonreduced gels (6, 17).

**Are Atypical Insulin Receptors an Artifact of Affinity Chromatography on Insulin-CDI-Agarose?** When insulin coupled to succinimidylpropionarnino-agarose (1) was used, and competition binding studies with 125I-labeled IGF-I and increasing concentrations of insulin, IGF-I, and MSA were performed on the eluate and unbound fractions, the results were the same as those obtained using insulin-CDI-agarose. This indicates that the CDI-agarose matrix per se was not responsible for the appearance of atypical insulin receptors in the column eluates.

The procedures associated with insulin affinity chromatography did not appear to generate atypical insulin receptors because these receptors were also present in wheat germ lectin-agarose-purified preparations. Most of the 125I-labeled MSA binding activity in the latter preparations could be ascribed to atypical insulin receptors rather than to crossreaction with classical insulin, IGF-I, or IGF-II receptors. First, in a representative experiment, 125I-labeled MSA binding (16% per 12 μg) was too high to be explained by crossreaction with insulin and/or IGF-I receptors (125I-labeled insulin binding and 125I-labeled IGF-I binding each being 42%). Second, 125I-labeled MSA binding to glycoproteins purified from wheat germ lectin-agarose was displaced by unlabeled insulin, IGF-I, and MSA in that order of potency and could be precipitated by antiserum B-10 IgG (30% at a final IgG concentration of 14 μg/ml). Finally, affinity crosslinking of 125I-labeled MSA to these glycoproteins revealed M₃, 140,000 reduced complexes and not the M₃, 260,000 monomers characteristic of the IGF-II receptor (data not shown).

**DISCUSSION**

The IGF-I binding activity copurifying with human placental insulin receptors can be ascribed to a small discrete population of atypical insulin receptors with moderate affinity for the insulin-like growth factors, IGF-I and MSA. It is not explained by crossreaction with classical insulin receptors or coelution of IGF-I or IGF-II receptors. It is likely that these atypical insulin receptors are the same as the type III IGF receptors detected by Hintz and colleagues (19) on human IM-9 lymphocytes and human placentals membranes by virtue of their specific 125I-labeled IGF-II binding, which was displaced almost equipotently by insulin, IGF-I, and IGF-II (19). Werther et al. (20) had noted earlier that specific 125I-labeled IGF-II binding to IM-9 lymphocytes was displaced far more readily by unlabeled insulin than by the IGFs. Although the atypical insulin receptors are distinct, they share many of the features of the insulin and IGF-I receptors. Their affinity for IGF-I is moderately high (Kd = 2 × 10⁻⁹ M) but 9-10 fold that of the IGF-I receptors (Fig. 2). Insulin is 5-15 times more potent than IGF-I in displacing 125I-labeled IGF-I from these sites (Fig. 1), indicating a high affinity for insulin (Kd = 0.2-0.6 × 10⁻⁹ M). They display intermediate crossreactivity toward polyclonal and monoclonal antibodies to the insulin and IGF-I receptors (Fig. 5) and have the same subunit structure as the insulin and IGF-I receptors (Fig. 6).

Like the IGF-I receptors in solubilized human placental membranes (16) and in the unbound fraction from the insulin affinity column (Fig. 5), only part of the 125I-labeled IGF-I bound to atypical insulin receptors could be precipitated by antiserum B-2. Similar results were obtained by using antiserum B-10 (Fig. 5). Thus, the atypical receptors must also contain subpopulations of receptors not recognized by these antisera. These receptors would contribute to a small degree to the two types of IGF-I binding sites present in solubilized human placental membranes and delineated on the basis of their immunoreactivity with antiserum B-2 (16). Since disulfide reduction of human placental IGF-I receptors results in the conversion of B-2 reactive receptors to B-2 nonreactive receptors (24), a similar mechanism could be invoked for the existence of B-2 nonreactive atypical insulin receptors.

Amino-terminal sequences have been obtained for both the α- and β-subunits of insulin receptors purified by desorption from insulin-Sepharose. Synthetic oligonucleotide probes based on these sequences were used by Ullrich et al. (21) to screen a placental cDNA library and identify a single cDNA clone encoding the entire human insulin receptor precursor. Sequencing and cloning studies were also performed by Ebina et al. (22) using insulin receptors purified by affinity
chromatography on a monoclonal antibody column (23). The latter preparation was reportedly contaminated by up to 20% with IGF-I receptors (22). Since atypical insulin receptors coelute with insulin receptors from insulin affinity columns and show more immunological relatedness to insulin receptors than to IGF-I receptors, it is likely that they contaminate purified receptor preparations and, in fact, account for the IGF-I receptors noted by Ebina et al. (22). cDNA probes for the insulin receptor gave multiple hybridization bands on RNA blot analysis (21, 22). These different mRNAs may represent modifications of the same gene (variable splicing, varying lengths of 3' or 5' untranslated sequences) but might also represent homologous genes, including one for the atypical insulin receptor. Also, since the nucleotide sequences for both reported receptor cDNAs are slightly different (21, 22), they could conceivably correspond to cDNA clones for the insulin receptor and the atypical insulin receptor.

The authors gratefully acknowledge the technical assistance of Ms. Alison Cox and the secretarial assistance of Linda Stafford. This work was supported by the National Health and Medical Research Council of Australia.