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Isolated Compared to Membrane-Bound Receptors Exhibit Altered Insulin/IGF Interaction

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Abstract—Insulin and insulin-like growth factors (IGFs) bind to their cognate receptors with high affinities, but due to their homology they may cross-react with each other's receptors. We performed a series of binding studies to reanalyze the cross-reactivity of insulin, IGF-I, and IGF-II to affinity-purified insulin (IR) and type 2 IGF receptors (IGF-2R) from human placental membranes. IR and IGF-2R were purified using insulin- and mannose-6-phosphate affinity chromatography (I-AC and M6P-AC). Binding studies were performed with ^{125}I-labeled and unlabeled ligands. According to immunoblotting, the only receptor species isolated by I-AC was IR, whereas the only receptor isolated by M6P-AC was IGF-2R. Isolated IR reacted to similar extent with ^{125}I-labeled insulin and ^{125}I-labeled IGF-II and significantly less with ^{125}I-labeled IGF-I, implicating predominance of IR-A. The affinity of IR towards heterologous ligands increased after its separation from other membrane proteins. Affinity-purified IGF-2R was almost unable to bind ligands under experimental conditions used in this work, but when incubated with ^{125}I-labeled ligands *prior to* affinity chromatography, IGF-2R interacted not only with IGF-II, but to a certain extent with the other two ligands. In the competitive M6P-AC, the binding of labeled ligands was inhibited with either homologous or heterologous ligands, in a dose dependent manner. In competitive ligand-blotting, specific interactions between ^{125}I-labeled insulin and IR, and ^{125}I-labeled IGF-II and IGF-2R were also inhibited with all unlabeled ligands, although to a different extent. The results presented in this work imply that isolation of IR an IGF-2R from their membrane milieu increases their reactivity towards all members of the insulin/IGF ligand family.

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Key words: insulin, insulin-like growth factors, receptors, affinity chromatography, ligand-binding studies

Insulin and insulin-like growth factors IGF-I and IGF-II are homologous peptides derived from a common ancestor and they share sequence identity with each other of approximately 45 to 50% of their amino acid residues [1]. Insulin is generally assumed to have metabolic actions, whereas IGFs have mostly mitogenic actions regulating cellular proliferation and growth. Despite the divergence in their biological functions, these peptides seem to maintain all their ancestral roles: IGFs are able to exert acute effects on metabolism and insulin is able to stimulate growth [2].

Close relation between insulin and IGFs is extended to their receptors and signaling pathways. The type 1 IGF receptor (IGF-1R) displays over 50% homology to insulin receptor (IR), having 84% homology in the tyrosine kinase domain of the β-subunit [3]. The biological effect of insulin is mediated after its binding to IR, whereas the effects of IGF-I and IGF-II are primarily mediated through their binding to IGF-1R. The type 2 IGF receptor (IGF-2R), also known as cation-independent mannose-6-phosphate receptor (CIM6P-R), is a single-chain polypeptide responsible for capturing IGF-II and its internalization and subsequent degradation [4].

Ligands bind to their cognate receptors with high affinities, but due to the homology between insulin and IGFs, as well as between IR and IGF-1R, insulin and IGFs cross-react with each other's receptor. IGFs interact with IR with approximately 100 times lower affinity than insulin (K_d ~ 0.1 nM), IGF-II being more reactive

Abbreviations: CIM6P-R, cation-independent M6P receptor; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; HyR, hybrid receptor; I-AC, insulin-affinity chromatography; IGF, insulin-like growth factor; IGF-1R and -2R, type 1 and 2 IGF receptors, respectively; IGFBP, IGF-binding protein; IR, insulin receptor; LBA, ligand-binding assay; M6P, mannose-6-phosphate; M6P-AC, M6P-affinity chromatography; PEG, polyethylene glycol.

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than IGF-I. IGF-I has several fold higher affinity for IGF-1R than IGF-II (K_d ~ 1 nM), while insulin binds with 10-100 times lower affinity [5]. IGF-2R is widely assumed to bind almost exclusively IGF-II (K_d ~ 1 nM) [6].

Besides receptors' affinity, the relative amount (concentration) of ligands and their availability (the ratio between free and protein-bound ligand) affect the ligand–receptor reactivity. Unlike insulin, which is produced and stored in pancreatic β-cells, IGFs are produced in many tissues, although mostly by the liver. The amount of IGFs in the circulation is 100-1000 times greater than that of insulin, but only a small portion (less than 1%) is free [1]. The rest is bound to IGF-binding proteins (IGFBP-1 to -6). Different tissues synthesize specific IGFBPs in variable ratios [7]. The overall reactivity of a ligand with a particulate receptor, therefore, depends on the specific conditions in a tissue, which are susceptible to modification. Part of the cellular signaling may be obtained via hybrid receptors (HyR), IR/IGF-1R, which further complicates the relation between these molecules [5].

Although affinities of a certain receptor towards homologous ligands vary significantly, and the cross-reactivity is generally assumed to be limited, our experimental results suggested it to be greater than expected. We, therefore, performed a series of binding studies to reanalyze the cross-reactivity of insulin, IGF-I and IGF-II to affinity-purified IR and IGF-2R from human placental membranes. In contrast to the most often used purification procedures that employ specific anti-receptor antibodies immobilized to a carrier resin, in this work the affinity purification of the receptors was achieved using gel-immobilized ligands (insulin and M6P) in order to isolate only functional, ligand-binding receptor species.

MATERIALS AND METHODS

Preparation of solubilized membranes. Human placentas were obtained after normal full-term deliveries from three healthy mothers, with the approval of the local Ethical Committee. Cell membranes were isolated after tissue homogenization and differential centrifugation, as previously described [8]. Solubilization of the membranes suspended in 0.05 M Hepes buffered saline, pH 7.4, was achieved by adding detergent Triton X-100 (final concentration 1% v/v) and stirring the mixture at 4°C for 1 h. The suspension was centrifuged at 100,000*g* at 4°C for 1.5 h and the supernatant containing solubilized membranes was stored in aliquots (10 mg of protein, 1 ml) at –50°C until used. The protein concentration in solubilizates was determined by the method of Bradford [9]. All experiments (in duplicate or triplicate) were performed with solubilized membranes from each placenta.

^{125}I-labeled ligands. Porcine insulin (Novo, Denmark), human IGF-I and IGF-II (GroPep, Australia) were labeled with ^{125}I by the chloramine T method [10]. The specific activities were approximately 100 μCi/μg. Labeled molecules were used within two weeks.

Insulin-affinity chromatography (I-AC). IRs were isolated from the solubilized membranes (10 mg of protein) using agarose-bound insulin (1 ml; Sigma-Aldrich, Germany). The solubilizate was perfused through the column for 1 h, left standing overnight, and again perfused for 1 h at 4°C. The unbound material was washed away with 20 ml of 0.05 M Tris-buffered saline, 0.1% Triton X-100, pH 7.4, and the specific elution was performed with 10 ml of 0.05 M acetate buffer, 1 M NaCl, 0.1% Triton X-100, pH 5.0 [11]. One-milliliter fractions were collected in test tubes containing 0.5 ml of 0.5 M Tris buffer, pH 7.4, and used in immunoblotting, affinity ligand-blotting, and ligand-binding assay.

Mannose-6-phosphate-affinity chromatography (M6P-AC). IGF-2Rs were isolated from the solubilized membranes (10 mg of protein) using agarose-bound M6P (1 ml; Sigma-Aldrich). The incubation procedure was the same as for I-AC. The unbound molecules were washed away with 20 ml of 0.05 M Hepes-buffered saline, 0.1% Triton X-100, pH 7.4, and bound proteins were eluted with 10 ml of 0.05 M Hepes-buffered saline, 1 M NaCl, 0.1% Triton X-100, pH 5.0 [12]. One-milliliter fractions were collected as described for the I-AC and tested in immunoblotting, affinity ligand-blotting, and ligand-binding assay. A variation of the M6P-AC included preincubation of the solubilized membranes (1 mg of protein) with ^{125}I-labeled ligands (10^5 cpm) at 4°C overnight before application to the column. The radioactivity was measured in eluted fractions. Competitive M6P-AC was performed with the solubilized membranes (1 mg of protein), ^{125}I-labeled ligands (10^5 cpm) and varying amounts of the unlabeled ligands (the amount of the unlabeled ligand in the incubation mixture ranged from 1 to 500 pmol).

Electrophoresis and immunoblotting. The samples (solubilized membranes and affinity-purified receptors) were subjected to SDS-PAGE (in a 6% gel), according to the method of Laemmli [13], and immunoblotting [14], employing monoclonal anti-IR (GroPep), anti-IGF-1R (Biosource, USA), anti-CIM6P/IGF-II-R primary antibodies (Calbiochem, USA), and HRP-conjugated anti-mouse IgG secondary antibody (Biosource). The receptors were visualized using enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, UK) followed by autoradiography (Kodak MXB film and developing reagents, France).

Ligand-binding assay (LBA). Membrane proteins in the fractions specifically eluted after affinity chromatography (0.1 ml) were incubated with ^{125}I-labeled ligands (0.1 ml, 10^5 cpm) in test tubes containing 0.3 ml of

0.05 M Hepes-buffered saline, pH 7.4, with gelatin (final concentration 0.2%) at 4°C overnight. Receptor complexes were precipitated by the addition of bovine IgG (0.1 ml, final concentration 0.05%) and 20% (w/v) polyethylene glycol (PEG) in 0.05 M phosphate-buffered saline, pH 7.4 (1.5 ml). After intensive vortexing, tubes were centrifuged at 4500*g* for 45 min, supernatants were aspirated, and the radioactivity of precipitates measured in a γ-counter. Competitive LBA, besides the above-mentioned constituents, included unlabeled ligands (in the range from 0.02 to 100 pmol per incubation mixture in 0.05 M Hepes-buffered saline, pH 7.4).

Affinity ligand-blotting [15]. Membrane proteins in the fractions specifically eluted after affinity chromatography (0.1 ml) were incubated with ^{125}I-labeled ligands (0.1 ml, 10^5 cpm, approximately 0.1 pmol) and, in competitive ligand-blotting, with a mixture of ^{125}I-labeled and unlabeled ligands (0.1 ml, 10 pmol) at 4°C for 24 h. A solution of 8 mM disuccinimidyl suberate (DSS) in dimethyl sulfoxide (DMSO) was added (0.1 ml) and, after incubation at 4°C for 15 min, the reaction was quenched by the addition of the Laemmli sample buffer. Receptor complexes were subjected to SDS-PAGE (in a 6% gel) and autoradiography.

RESULTS

Insulin and IGF receptors bound to cell membranes are surrounded by a number of molecules that may influence their interaction with ligands. Isolation of these receptors, especially ligand-affinity purification as employed in this work, enables direct investigation of a very specific ligand–receptor interaction, undisturbed by the presence of other membrane molecules.

Proteins from the solubilizates that were chromatographed on affinity columns were separated into unbound molecules and those that specifically reacted with the immobilized ligand—insulin or M6P. Immunoblotting of proteins in the third unbound and in the third specifically eluted fraction was performed in order to detect IR, IGF-1R, and IGF-2R. Figure 1 shows the representative blots.

The unbound fraction obtained upon I-AC contained all analyzed receptors, whereas the eluted fraction contained only IR, according to its immunoreactivity with anti-IR antibody (Fig. 1a). Similarly, after M6P-AC, all three types of receptors were found in the unbound fraction, but the IGF-2R was the only one recognized in the specific eluate (Fig. 1b). Thus, placental IR and IGF-

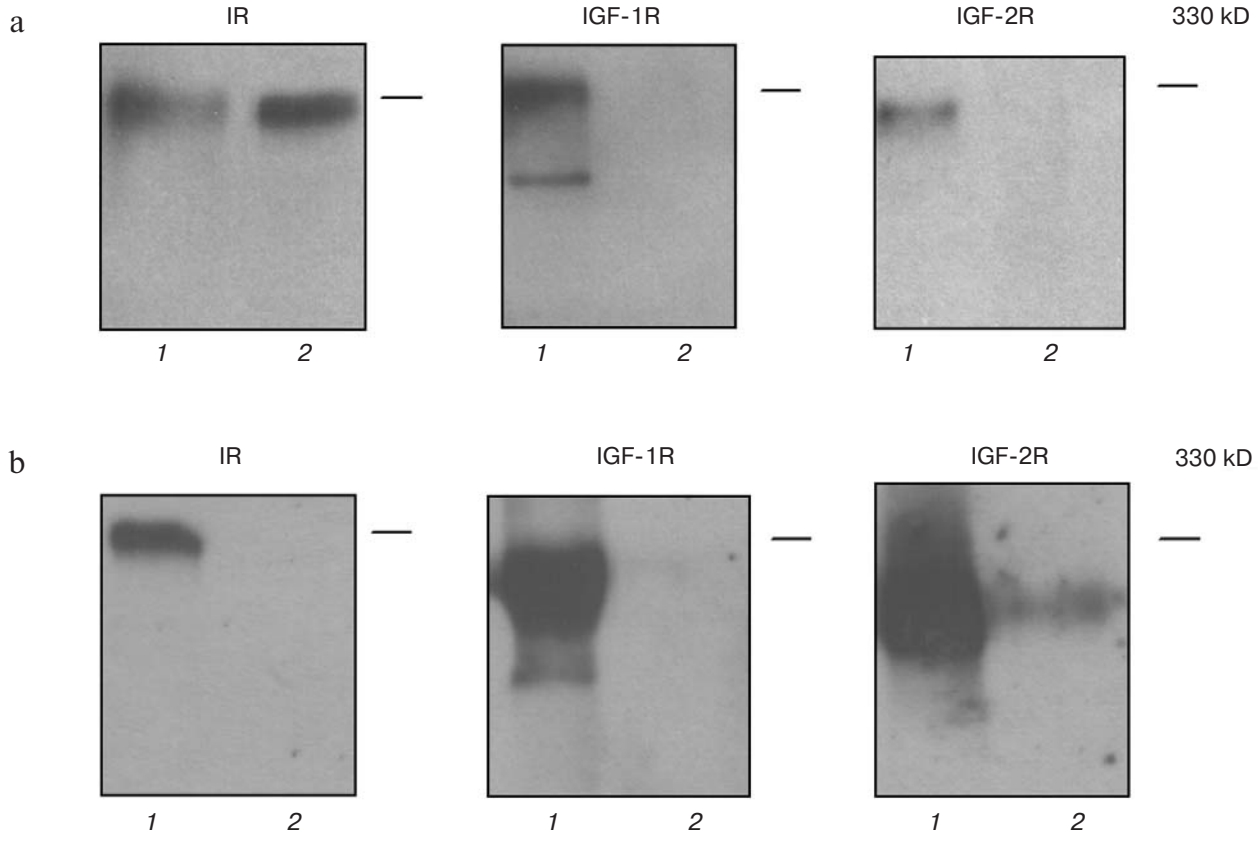

Fig. 1. SDS-PAGE and immunoblotting of the unbound (*1*) and specifically eluted proteins (*2*) separated by insulin- (a) and mannose-6-phosphate- (b) affinity chromatography. Molecular mass marker indicated on the right hand side is thyroglobulin monomer (330 kD).

2R were separated from other receptors by the affinity chromatography.

LBA was performed with specifically eluted fractions and the results are presented as bound radioactivity corrected for nonspecific binding in Fig. 2. Placental IR interacted to similar extent with ^{125}I-labeled insulin and ^{125}I-labeled IGF-II, whereas its reactivity with ^{125}I-labeled IGF-I was much lower (Fig. 2a). In contrast, almost no ligand binding was detected in LBA that followed M6P-AC (Fig. 2b). Two possible reasons could be responsible for this finding; either the IGF-2R lost its ligand-binding activity or the PEG method used in the LBA was not appropriate to precipitate ligand–IGF-2R complexes. In order to test these hypotheses, in separate experiments, solubilized membranes were preincubated with radioligands *prior to* M6P-AC. As expected, the strongest interaction detected was of the IGF-2R with ^{125}I-labeled IGF-II, while ^{125}I-labeled IGF-I reacted to some extent. Unexpectedly, we detected ^{125}I-labeled insulin binding (Fig. 2c).

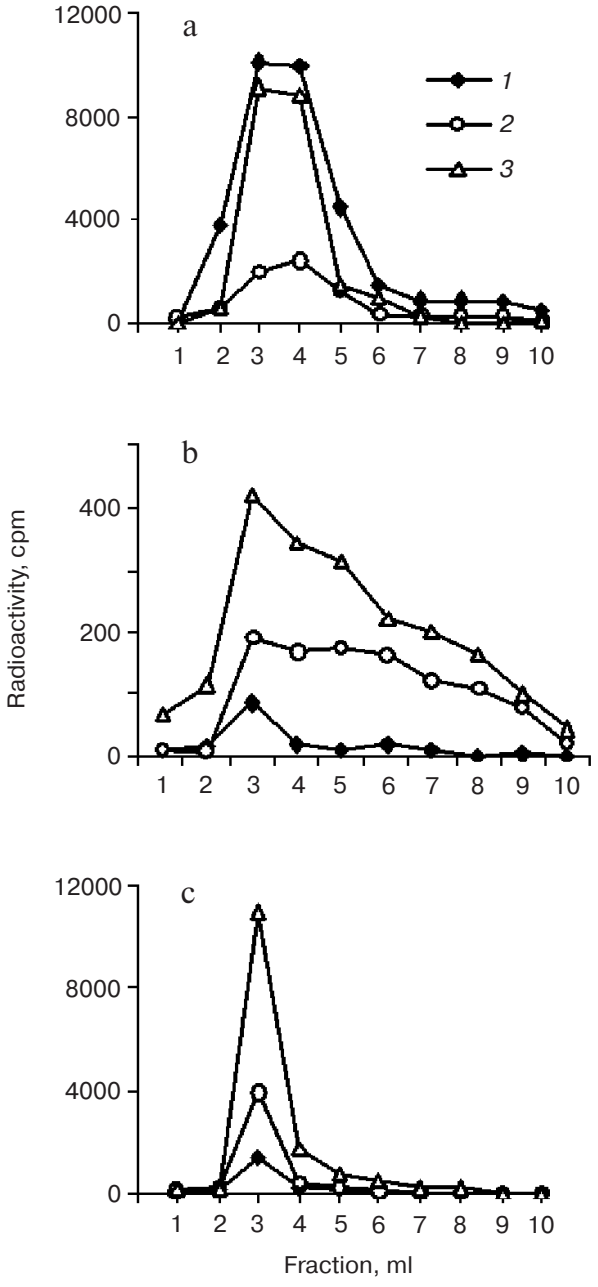

Fig. 2. Ligand-binding assays of receptors separated by insulin- (a) and mannose-6-phosphate- (b and c) affinity chromatography. ^{125}I-labeled ligands—insulin (*1*), IGF-I (*2*), and IGF-II (*3*)—were incubated with isolated receptors after affinity chromatography (a and b) or with solubilized membranes *prior to* affinity chromatography (c).

Coefficient of variation (CV) in the binding assays between samples originated from the same placenta ranged from 3.6 to 4.4%. The CVs obtained between the samples from different placentas were up to 20%, depending on the radioligand used. The reactivities of IR and IGF-2R with different ligands (estimated from the peak heights) were preserved for all placentas.

In order to quantify ligand-binding parameters of the isolated receptors and to compare them with the results that were previously obtained with the membrane solubilizates [16], the appropriate competitive assays in the presence of unlabeled ligands were performed. IC_{50} value (the amount of unlabeled ligand that inhibits 50% of the maximal binding) was calculated for the specific receptor/labeled ligand/unlabeled ligand analytical system.

IC_{50} values for competitive LBA performed with solubilized membranes or isolated IR and different ligand combinations are summarized in the table. IC_{50} for IR and ^{125}I-labeled/unlabeled insulin ligand pair was almost the same whether this receptor was isolated or present in the membrane solubilizate. IC_{50} was however lower for the isolated IR than in the solubilizate for both ^{125}I-labeled insulin/unlabeled IGF-I and ^{125}I-labeled insulin/unlabeled IGF-II ligand pairs, suggesting increased affinity of the IR for IGFs when separated from the rest of the membrane constituents. The binding of ^{125}I-labeled IGF-II to the isolated IR was most potently inhibited by IGF-II and less efficiently with the other two ligands. Similarly, the binding of ^{125}I-labeled IGF-I was most potently inhibited by IGF-I, followed by IGF-II and insulin.

In competitive M6P-AC, IC_{50} represented the amount of unlabeled ligand that caused 50% reduction in the maximal binding of radioligand/IGF-2R complexes to immobilized M6P. These IC_{50} values cannot be directly compared to those obtained in LBA, due to completely different analytical method, experimental conditions, and relative concentration ratios between reactants. IC_{50} values calculated for competitive M6P-AC can only be compared among themselves, to illustrate the relative effect of a particular ligand. In the homologous competitive M6P-AC, the following IC_{50} values were found: 164 ± 9 pmol for ^{125}I-labeled/unlabeled insulin pair, 12 ±

IC_{50} values for IR in membrane solubilizates and in isolated IR preparations

^{125}I-Labeled ligand		IC_{50} (mean ± SD), pM		
	Ligand	insulin	IGF-I	IGF-II
^{125}I-Labeled insulin	solubilizate	175 ± 8	not reached	8720 ± 673
	IR	162 ± 6	6560 ± 1115	1958 ± 49
^{125}I-Labeled IGF-I	IR	21430 ± 1531	1703 ± 131	7021 ± 1225
^{125}I-Labeled IGF-II	IR	22490 ± 1730	28820 ± 2620	6700 ± 1340

2 pmol for ^{125}I-labeled/unlabeled IGF-I pair, and 2.7 ± 0.3 pmol for ^{125}I-labeled/unlabeled IGF-II pair. In the heterologous competitive M6P-AC, 50% inhibition of ^{125}I-labeled IGF-II binding was achieved in the presence of 45 ± 7 pmol of unlabeled IGF-I, whereas unlabeled insulin, even when 500 pmol was used, was not able to displace ^{125}I-labeled IGF-II to 50% of the initial binding. These results confirmed that isolated IGF-2R can bind IGF-I and insulin, besides IGF-II. The binding of labeled ligands can be inhibited with both homologous and heterologous ligands in a dose dependent manner, although to a different extent.

Cross-reactivity of the isolated receptors with all ligands was also demonstrated in affinity ligand-blotting. Competitive ligand-blotting using 100 times more unlabeled than labeled ligand showed that specific interactions between ^{125}I-labeled insulin and IR (Fig. 3a) and ^{125}I-labeled IGF-II and IGF-2R (Fig. 3b) can be inhibited with all unlabeled ligands, again with different efficiency.

DISCUSSION

Placental cells possess IR, both types of IGF-Rs as well as HyR composed of IR and IGF-1R hemireceptors [17]. According to the classical view, in physiological situations insulin and IGFs bind preferentially to the receptors of the highest affinity. In some tissues, including placenta, HyRs are the most represented receptor type, the percent of HyRs exceeding that of individual IR and IGF-1R [5]. The human IR exists in two isoforms, IR-A and IR-B, generated by alternative splicing of the IR gene [18]. IR-A functions as a high-affinity receptor for IGF-II and is implicated in fetal growth and cancer biology [19, 20]. Activation of IR-A by insulin leads primarily to metabolic effects, whereas activation of IR-A by IGF-II leads primarily to mitogenic effects [21]. Each of the two isoforms is equally potent in making HyRs with IGF-1R, but the hybrids have different functional characteristics [5]. HyR^B has a high affinity only for IGF-I and behaves more like IGF-1R than IR. HyR^A has even higher affinity for IGF-I and, according to Frasca et al., also binds insulin and IGF-II [19]. On the contrary, Slaaby et al. found that HyRs have a very low affinity for insulin, irrespective of the IR variant [22]. Although the precise roles of the two types of HyRs are not clear, the cross-reactivity of ligands with these receptors deserves attention as this may influence metabolic/mitogenic equilibrium in target tissues.

In this work, we describe the cross-reactivity of insulin, IGF-I, and IGF-II with isolated IR and IGF-2R originating from human placental membranes. According to the immunoblotting, the only receptor species isolated by the I-AC was IR. IGF-1R and IGF-2R, also present in the solubilized membrane preparations, were washed away with the unbound material. Non-reactivity of the

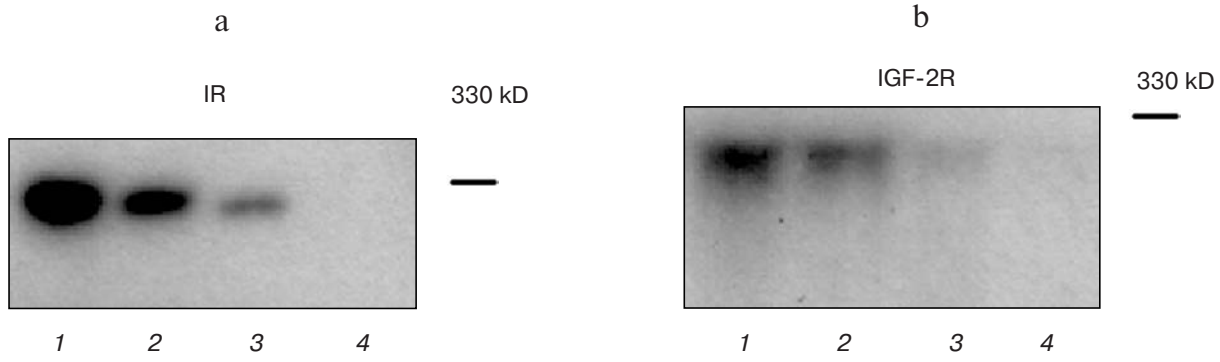

Fig. 3. SDS-PAGE of the affinity labeled IR with ^{125}I-labeled insulin (a) and IGF-2R with ^{125}I-labeled IGF-II (b). a) Maximal affinity labeling of IR (*1*) was gradually inhibited with unlabeled IGF-I (*2*), IGF-II (*3*), and insulin (*4*). b) Maximal affinity labeling of IGF-2R (*1*) was gradually inhibited with unlabeled insulin (*2*), IGF-I (*3*), and IGF-II (*4*).

receptor fraction specifically eluted from I-AC with anti-IGF-1R antibodies suggested the absence of HyRs. Therefore, IRs were separated from other types of receptors on the immobilized insulin column. These IRs bound similarly trace quantities of ^{125}I-labeled insulin and ^{125}I-labeled IGF-II, but much less ^{125}I-labeled IGF-I, which strongly suggests the predominance of IR-A isoform in the IR preparation isolated in this work.

The only receptor species identified by the immunoblotting of the fraction specifically eluted upon M6P-AC was IGF-2R. IR and IGF-1R were present in the unbound fraction. In LBA, however, the reactivity of the isolated IGF-2R was very low with all ligands tested. Denaturation of the IGF-2R or inappropriate complex precipitation may be responsible for such results. PEG, as precipitating agent, is proposed for pelleting globular proteins, while IGF-2R is known to have elongated conformation [23]. Thus, the analytical system used in this study may not be optimal to precipitate ligand–IGF-2R complexes. Moreover, IGF-2R binds ligands at neutral pH [24] and, although our experimental conditions were designed to correct acidic pH immediately after the elution, the reactivity of IGF-2R could be disturbed even during the short-term exposition to the low pH. Linnell and colleagues analyzed the effect of pH on IGF-II binding to several IGF-2R domain constructs [25]. Decreasing pH did not significantly affect the association of IGF-II with domains 1-15 until pH 5.5, when the association rate was decreased 28-fold. No binding was detectable at pH 5.0. The association rate returned to the initial value after the elevation of pH, but the dissociation rate was also significantly higher [25].

The presence of the immobilized M6P is another variable in our system that has to be considered. IGF-2R was demonstrated to form oligomeric complexes in the absence of M6P ligands [26]. The occupancy of the M6P-binding site, even by a low-affinity monovalent ligand, affects the conformation of IGF-2R and its ability to bind IGF-II. In order to detect whether the functional IGF-2R molecules were present, membrane solubilizates were incubated with ^{125}I-labeled ligands prior to M6P-AC. Specifically eluted fractions contained complexes of radioligands with IGF-2R. Besides expected complexes [^{125}I]IGF-II–IGF-2R, also [^{125}I]IGF-I– and even [^{125}I]insulin–IGF-2R complexes were detected in the column eluates. The possible contamination of the preparation with other types of receptors is excluded, as neither IR nor IGF-1R, actually, possess M6P-binding domains in their structure.

To quantify the alteration in binding parameters of the isolated receptors, IC_{50} values for different receptor–ligand analytical systems were calculated. In general, the affinity of the IR for heterologous ligands increased after its separation from other membrane proteins, whereas isolated IGF-2R was capable of IGF-I and insulin binding.

Taken together, results presented in this work suggest greater cross-reactivity between homologous ligands: insulin, IGF-I, and IGF-II and their isolated receptors than is generally described in the relevant literature. The differences in their binding can be described in terms of "severalfold", but not "several orders of magnitude".

Investigators that determined affinities of insulin and IGF receptors, including ourselves [8], routinely utilized solubilized membranes as a source of receptors, and competitive ligand-binding assays as a method. The solubilized membranes contain all types of receptors and, to a certain extent, binding proteins. IGFBP-1 (25 kD) was found to be present in the placental solubilizates even after extensive washing of the membranes before solubilization [27]. IR and IGFRs separated from smaller molecules by the size exclusion chromatography, in competitive LBA demonstrated altered affinity for their ligands (data not shown). Although the size exclusion chromatography enabled us to separate the receptors from the IGFBP-1, it was ineffective in the separation of IR from the IGFRs.

The specific receptors can be isolated by affinity chromatography, designed as ligand-affinity chromatography, where only ligand-reactive receptors are expected to be extracted from the receptor pool. Both types of the receptors, whose isolation is described in this work, were able to recognize and bind all three ligands, although to a different extent. The structural homology of insulin, IGF-I, and IGF-II, therefore, enables them all to be recognized by insulin and IGF receptors, but the intensity of the specific interaction (and the following binding constants including affinity), actually, depends on the presence of other interfering factors, including ligands, receptors, binding proteins and, perhaps, other molecular species.

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