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THE LIGAND BINDING SUBUNIT OF THE INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR HAS PROPERTIES OF A PERIPHERAL MEMBRANE PROTEIN

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SUMMARY: ^{125}I-insulin-like growth factor 1 was cross-linked to its receptor in human placenta microsomal membranes. The microsomes were treated with urea, with dithiothreitol or with both reagents prior to centrifugation at 100,000 x g. We found that >80% of the label was membrane-associated following separate treatment with urea or dithiothreitol, but >80% of the radioactivity remained in the supernatant after simultaneous exposure to both reagents. In identical experiments employing ^{125}I-epidermal growth factor, no condition led to the release of >10% of label from the membrane. We conclude that the ligand binding subunit of the insulin-like growth factor 1 receptor, like peripheral membrane proteins, lacks a membrane anchoring domain.

On the bases of several independent criteria, the receptors for insulin (1-3) and insulin-like growth factor one (IGF-1) (4-6) have been determined to have the same tetrameric subunit structure, namely a disulfide-linked complex consisting of two α subunits (M_r 130,000) that recognize and bind ligand and two β subunits (M_r 90,000). The β subunit of the insulin receptor (7-11) possesses intrinsic, tyrosine-specific protein kinase activity as does the β subunit of the IGF-1 receptor (12-13). The amino acid sequence of the insulin receptor predicted from the nucleotide sequence of cloned receptor cDNA (14,15) confirmed the compositional data previously obtained (1-3) and showed that only the β subunit had a likely transmembrane sequence. Previous indirect studies have shown that only the β subunit of the insulin receptor could be iodinated vectorally from the cytoplasmic side, i.e. that little if any of the α subunit resided in the cell cytoplasm (16). A recent study

ABBREVIATIONS: Insulin-Like Growth Factor, IGF; Epidermal Growth Factor, EGF; Dimethylsulfoxide, DMSO; Phenylmethanesulfonylfluoride, PMSF; Dithiothreitol, DTT.

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directly demonstrated that the α subunit of the insulin receptor could be released from membranes by reducing receptor disulfide bonds under denaturing conditions (17). In the present case, we demonstrate that the α subunit of the IGF-1 receptor, like the closely related insulin receptor α subunit, lacks a membrane anchoring domain.

MATERIALS AND METHODS

Membrane Preparations: Microsomal membranes from human placenta were obtained as previously described (9,18). Briefly, term placentas obtained following Ceasarian sections were rinsed in ice-cold PBS to remove as much blood as possible and were then cut into pieces and homogenized in 4-5 batches using a Tekmar "Tissuemizer". The homogenization buffer consisted of 30 mM Hepes, 25 mM benzamidine, 0.25 M sucrose, 1 mM PMSF, 1 mM 1,10 phenanthroline, 50 units of aprotinin, 10 μM leupeptin, 1 μM pepstatin adjusted to pH 7.6 and kept at 4 °C. Subsequent steps were exactly as previously outlined (9,18).

Affinity Cross-linking and Membrane Treatment: ^{125}I-IGF-1 (1 nM) was incubated with placental microsomes for 30 min at 25 °C in 30 mM Hepes, pH 7.6. After equilibrium binding was reached, the membrane-receptor complex was placed on ice and 10 mM disuccinimidyl suberate in DMSO was added at a 1:50 dilution to a final concentration of 0.2 mM. Cross-linking was terminated with the addition of 1.5 M Tris, pH 8.8 at a 1:10 dilution. The microsomes were then exposed to urea, to dithiothreitol or to both reagents for 45 min at room temperature and were then centrifuged for 1 hour at 100,000 x g. The resultant pellet was resuspended in a volume of 30 mM Hepes equal to that of the supernatants and equal aliquots of supernatant and resuspended pellet were electrophoresed according to the method of Laemmli (19). The same protocols were employed to cross-link insulin and epidermal growth factor (EGF) to their respective receptors and to analyze their membrane association following reagent exposure.

Materials: Hepes and dithiothreitol were obtained from Research Organics. Disuccinimidyl suberate was obtained from Pierce Chemicals. Leupeptin and pepstatin were purchased from Vega Biochemicals. ^{125}I-IGF-1 was obtained from Amersham, and unlabeled IGF-1 was obtained from Amgen. ^{125}I-EGF was purchased from ICN and unlabeled growth factor from Calbiochem. All other reagents were obtained from Sigma Chemical Co.

RESULTS AND DISCUSSION

Previous data documenting the effects of reductants on insulin receptor structure indicated that denaturing conditions were necessary to separate α subunits from β subunits (18,20). Receptor preparations in membranes or in non-ionic detergents can be treated with as much 50 mM dithiothreitol, and then upon removal of reductant, analysis by SDS-PAGE revealed the exclusive receptor form to be an Mr 210,000 species, the αβ receptor half (18,20). These data suggested that the α-β linkage is not solely dependent on disulfide bridges, but rather that these disulfide bridges were inaccessible to reduction unless denaturants were present. Recent experiments showing that the α

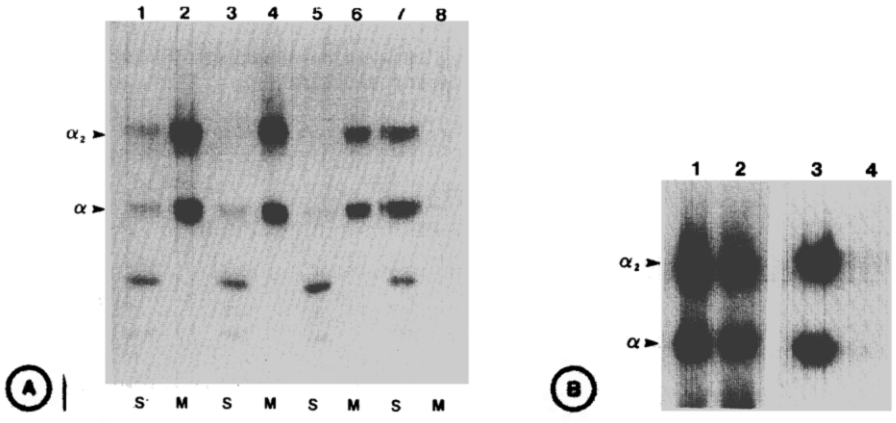

Figure 1A. Effect of Dithiothreitol and Urea on the Membrane Association of the IGF-1 receptor α subunit.

Approximately 1 ml of placental microsomes at 5 mg/ml protein were cross-linked to ^{125}I-IGF-1 as described in Materials and Methods. This preparation was divided in four 0.2 ml parts and diluted with an equal volume of 30 mM Hepes, pH 7.6 (lanes 1 and 2), 80 mM dithiothreitol (DTT) in Hepes (lanes 3 and 4), 8 M urea (lanes 5 and 6) and 80 mM DTT in 8 M urea in Hepes (lanes 7 and 8). After 45 min, the preparations were centrifuged to separate membrane and pellet, and following resuspension of pellets in 0.4 mls 30 mM Hepes, pH 7.6, 100 µl aliqouts were used for electrophoresis (19). Depicted is an autoradiogram of a stained and dried 7.5 % acrylamide gel.

Figure 1B. Specificity of ^{125}I-IGF-1 afinity labeling.

1 nM ^{125}I-IGF-1 was crosss-linked to placental microsomes as described above and in Materials and Methods either alone (lanes 1 and 3) or in the presence of 8 µM unlabeled insulin (lane 2) or 0.25 µM unlabeled IGF-1. 100 µl were run on a 7.5 % acrylamide gel.

subunit of the insulin receptor could be released from membranes only after simultaneous treatment of membranes with reductant and denaturant further supported this notion (17). Much less information is available concerning the structure of the IGF-1 receptor. Thus, we sought to determine if the ligand binding α subunit of the IGF-1 receptor can also be released from membranes following their exposure to reducing agents and denaturants.

Figure 1, panel A, shows the results of an experiment where ^{125}I-IGF-1 was affinity cross-linked to its receptor in placental microsomes and then treated with the reagents indicated prior to centrifugation, SDS-PAGE and autoradiography. It is clear that only traces of labeled α subunit appear in the supernatant after exposure to either dithiothreitol or to urea. However, when these reagents are present together (lanes 7 and 8), essentially all the α subunit is found in the supernatant (see also Table 1). To verify that we were in fact dealing with the IGF-1 receptor and not the insulin receptor,

TABLE 1

Quantitation of ^{125}I-ligand partitioning between supernantant (S) and membrane (M) following reagent exposure

	LIGAND					
	IGF-1		EGF		Insulin	
TREATMENT	S	M	S	M	S	M
Nothing	12	88	10	90	0	100
Dithiothreitol (DTT)	20	80	10	90	25	75
Urea	12	88	11	89	20	80
Urea + DTT	84	16	11	89	78	22

Ligands were cross-linked to receptors, centrifuged and electrophoresed exactly as described in figures 1 and 2. Following staining, drying and autoradiography of the gels, the band(s) corresponding to receptor were cut out and counted in a gamma counter. The data are expressed as percent of total partitioned between supernatant and membrane. The total counts for any condition (membrane + supernatant) were ± 20% for a given experiment. The results of single experiments are shown for insulin and EGF, and the IGF-1 data are the mean of two experiments.

we performed competition experiments as shown in panel B. Cross-linking of ^{125}I-IGF-1 to its receptor is completely blocked by excess unlabeled IGF-1 (lanes 1 and 2) but is essentially unaffected by the presence of excess unlabeled insulin (lanes 3 and 4). In these experiments, a large amount of α-α cross-linking also occured as is visible near the top of the gel. In a separate experiment (not shown) we determined that this species has an apparent Mr of 250,000 and migrates substantially slower than αβ halves. Thus, these data support the notion that the α subunit of the IGF-1 receptor lacks a transmembrane anchoring region.

To ascertain that the relatively harsh treatment of membranes with dithiothreitol and urea did not lead to a general release of proteins with a single transmembrane spanning region, we cross-linked ^{125}I-EGF to its receptor in placental microsomes and treated these membranes as in figure 1A prior to electrophoresis and autoradiography. The EGF receptor is known to have an extracellular binding domain and an intracellular kinase domain connected by a single hydrophobic sequence (21). As shown in figure 2, there was no release of label into the supernatant even when urea and dithiothreitol were employed together to effect solubilization of the ligand binding domain of the EGF

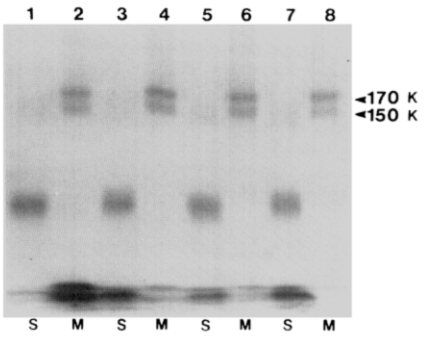

Figure 2. Effect of Urea and Dithithreitol and the Membrane Association of ^{125}I-EGF Cross-linked to its Receptor.

All conditions were exactly as described in Figure 1A. 1nM ^{125}I-EGF was employed to label the EGF receptor.

receptor. A quantitiation of the experiments shown in the first two figures plus an additional experiment employing insulin is shown in Table 1. Clearly, the binding subunits for the insulin and IGF-1 receptors are released into the supernatant by both reagents whereas this treatment is not effective for EGF.

These data document another structural feature of the IGF-1 receptor that is identical to the same feature of the insulin receptor, namely the entirely extracellular disposition of the ligand binding subunit. For both receptors, the interaction of an α subunit with a β subunit that leads to activation of kinase activity must therefore be initiated outside the cell because this is where the entire ligand binding domain resides.

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