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Structure of the insulin-like growth factor receptor in chicken embryo fibroblasts

(multiplication-stimulating activity/insulin/disuccinimidyl suberate/affinity crosslinking)

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ABSTRACT **The insulin-like growth factors (IGFs) and insulin stimulate DNA synthesis and cell multiplication in chicken embryo fibroblasts in culture. This response appears to be mediated by interaction with a single type of IGF receptor. The present study examines the subunit structure of this receptor by covalently crosslinking two ^{125}I-labeled IGFs, IGF-I and multiplication-stimulating activity (MSA), to chicken embryo fibroblasts by using disuccinimidyl suberate. After solubilization, NaDodSO$_4$/polyacrylamide gel electrophoresis, and autoradiography, IGF receptor complexes of appropriate specificity were identified; they had M_r ≈130,000 (major band) and ≈260,000 (minor band) under reducing conditions and M_r >300,000 without disulfide reduction. The proportion of the M_r 260,000 component increased with increasing concentration of crosslinking agent, suggesting that it was formed from smaller proteins during the crosslinking procedure. The IGF receptor in chicken embryo fibroblasts resembles the insulin receptor in size and structure but can be distinguished by a higher affinity for IGF-I and MSA than for insulin. Although IGF receptors with different structure and specificity have been recognized in other tissues, the function of these binding sites is unknown. The present study demonstrates that the IGF receptor of chicken embryo fibroblasts that appears to mediate the growth-promoting effects of the IGFs contains a M_r ≈130,000 binding subunit and exists as a native receptor complex of M_r >300,000.**

The insulin-like growth factors (IGFs) or somatomedins are a family of polypeptides with growth-promoting effects in cultured fibroblasts, insulin-like activity in adipocytes *in vitro*, and anabolic effects in chondrocytes *in vitro* (1). Somatomedins with similar biological properties and similar interactions with specific receptors and serum carrier proteins have been purified from human and rat plasma and from culture medium conditioned by a cell line (BRL 3A) derived from normal rat liver [multiplication-stimulating activity (MSA)] (1–8, **). By chemical structure and immunologic and receptor reactivities, these polypeptides fall into two subclasses: IGF-I-like peptides [including IGF-I (2), basic somatomedin (3), somatomedin C (5), and rat somatomedin**] and IGF-II-like peptides [including IGF-II (1, 2) and MSA (6–8)]. Plasma levels of the IGF-I-like peptides show more marked growth hormone dependence than do the IGF-II-like peptides (9).

Like other polypeptide hormones, the IGFs are thought to initiate their biological actions on target tissues by binding to specific cell surface receptors (1, 10). Using different ^{125}I-labeled IGFs as radioligands, we have distinguished different types of IGF receptors (10–12). Although IGF receptors generally recognize both IGF-I-like and IGF-II-like peptides, receptors in different tissues and even in the same tissue can be distinguished by differences in their affinity for IGF-I and IGF-II and by their reactivity with insulin (10–13). This difference in binding specificity appears to correlate with the subunit structure of the IGF receptors (14). Using covalent crosslinking of ^{125}I-labeled IGF-I and MSA to receptors on rat liver membranes and BRL 3A2 cells, we demonstrated two distinct IGF-receptor structures: (*i*) a M_r ≈260,000 complex (reduced), not linked by disulfide bonds to other membrane proteins, whose formation was not inhibited by insulin; and (*ii*) a predominant M_r ≈130,000 complex (reduced), linked by disulfide bonds to other membrane proteins in a native complex of M_r >300,000, whose formation was inhibited by insulin (14).

The existence of two types of IGF receptor structures raises the question of whether these receptors mediate the same or different biological functions. Because many tissues possess both types of IGF receptors, it is difficult to resolve which effect is mediated by which receptor. Chicken embryo fibroblasts provide a unique opportunity to address this question. Competitive binding studies indicate that ^{125}I-labeled IGF-I, IGF-II, and MSA bind to the same receptor in chicken embryo fibroblasts; there is no evidence that these ligands bind to a second IGF receptor with a different specificity (12). IGF-I, IGF-II, and MSA stimulate DNA synthesis and cell multiplication in chicken embryo fibroblasts (11, 15–17). The growth-promoting effects of the IGFs in chicken embryo fibroblasts appear to be mediated by the single IGF receptor identified in binding experiments. IGF-I, IGF-II, MSA, insulin, and proinsulin inhibit binding to this receptor and stimulate DNA synthesis with the same concentration dependence and the same relative potencies (1, 12, 15–19).

In the present study, we used covalent crosslinking with disuccinimidyl suberate to determine the subunit structure of the chicken embryo fibroblast IGF receptor, an IGF receptor with defined biological function.

MATERIALS AND METHODS

Chicken embryo fibroblast cultures were established from 12-day embryo carcasses (19). Binding experiments were performed 3–5 days after plating of tertiary cultures in 35-mm Lin-

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Abbreviations: IGF, insulin-like growth factor; MSA, multiplication-stimulating activity.

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bro cluster plates as described (18, 19). The cell monolayers ($\approx 1 \times 10^6$ cells per well) were washed twice with Dulbecco's phosphate-buffered saline. The washed cells were incubated for 3 hr at 22°C with 0.8 ml of Hepes pH 8.0 binding buffer (14) containing ^{125}I-labeled MSA (^{125}I-MSA) (15 ng/ml) or ^{125}I-labeled IGF-I (^{125}I-IGF-I) (5 ng/ml) or for 3 hr at 15°C with ^{125}I-labeled insulin (^{125}I-insulin) (25 ng/ml); unlabeled polypeptides were added at the indicated concentrations.

Affinity crosslinking was performed as described (14). Disuccinimidyl suberate was freshly dissolved in dimethyl sulfoxide and added at a final concentration of 0.1 mM unless indicated otherwise. The cells were solubilized with $NaDodSO_4$, the lysate was subjected to slab gel electrophoresis [5% polyacrylamide and discontinuous buffer system of Laemmli (20)], and the gels were autoradiographed as described (14).

Unlabeled and ^{125}I-labeled polypeptides and chemicals were as described (14).

RESULTS

When ^{125}I-IGF-I, ^{125}I-MSA, and ^{125}I-insulin were incubated with chicken embryo fibroblasts, covalently crosslinked to the cells, and the products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis without reduction, one major and one minor band of M_r >300,000 were specifically labeled (Fig. 1). The migration of these two bands was similar for each of the three ligands. Because of the absence of reliable protein markers in this M_r range, it is difficult to estimate the M_r of these two bands precisely.

When $NaDodSO_4$/polyacrylamide gel electrophoresis was performed after reduction with dithiothreitol, each of the three ligands gave a major band of $M_r \approx 130{,}000$ and a minor band of $M_r \approx 260{,}000$ (Fig. 2). The radioactivity associated with these bands was abolished by high concentrations of the same unlabeled polypeptides. These M_r assignments are only approximate because the receptors are almost certainly glycoproteins, and glycoproteins migrate anomalously on $NaDodSO_4$/polyacrylamide gel electrophoresis relative to nonglycoprotein standards.

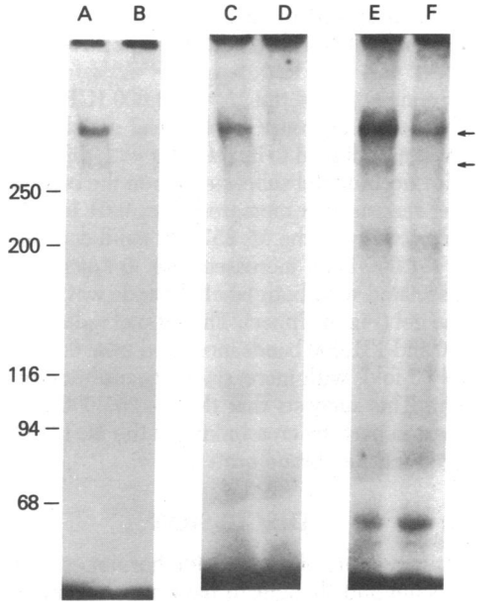

FIG. 1. Autoradiogram showing ^{125}I-IGF-I (lanes A and B), ^{125}I-MSA (lanes C and D), and ^{125}I-insulin (lanes E and F) receptor complexes crosslinked to chicken embryo fibroblasts and examined without reduction. Fibroblasts were incubated in the absence (lanes A, C, and E) and in the presence (lanes B, D, and F) of the appropriate unlabeled compound (0.5, 1, or 10 μg/ml, respectively). Samples were crosslinked, treated with 5 mM iodoacetamide, and solubilized in $NaDodSO_4$ without dithiothreitol. In this experiment, ^{125}I-insulin also appeared in two minor bands of apparent $M_r \approx 200{,}000$ and $\approx 65{,}000$ (lane E); formation of these bands was not inhibited by unlabeled insulin (lane F) and they presumably represent nonspecific binding. The presence of a visible band of decreased intensity corresponding to the upper arrow in lane F may reflect the longer time of exposure for this gel. M_r are shown $\times 10^{-3}$.

To determine whether the three radioligands were crosslinked to different binding sites, the specificity of formation of these radioactive complexes was examined. The ability of different concentrations of IGF-I, MSA, and insulin to inhibit ^{125}I-IGF-I complex formation is shown in Fig. 3. Under reducing conditions, ^{125}I-IGF-I radioactivity was specifically associated with a major band of M_r 131,000 ± 2600 and a minor band of M_r 259,000 ± 5100 (mean ± SD; $n = 3$). The labeling of both bands was decreased by incubation with IGF-I at 10 ng/ml and abolished by IGF-I at 0.5 μg/ml. By contrast, insulin at 10 ng/ml and MSA at 10 ng/ml had little effect on the labeling of these two bands. However, MSA was more potent than insulin in competing for ^{125}I-IGF-I binding: at 100 ng/ml and 1 μg/ml it decreased the radioactivity in both bands more completely than did the same concentrations of insulin. Thus, the specificity of formation of both bands is appropriate for an IGF–receptor complex.

^{125}I-MSA also appeared to bind to an IGF–receptor complex of similar size and specificity (Fig. 4). ^{125}I-MSA radioactivity was specifically associated with bands of M_r 131,000 ± 1200 and 257,000 ± 5000 (mean ± SD; $n = 3$). The M_r 131,000 band was labeled more intensely. The labeling of both bands was progressively decreased and finally abolished by incubation with increasing concentrations of MSA (10 ng/ml to 1 μg/ml). Unlabeled insulin also decreased the radioactivity associated with these two bands in a dose-dependent manner; however, insulin was slightly less potent than MSA in inhibiting the labeling of these two bands (for example, at 300 ng/ml). The similarity of potencies of MSA and insulin in inhibiting the binding of ^{125}I-

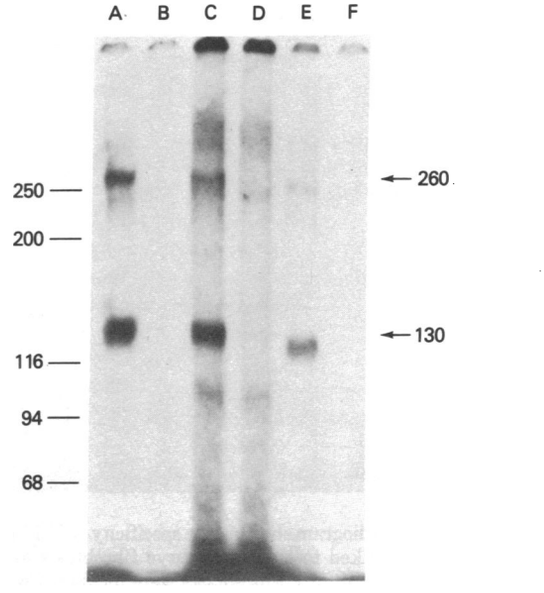

FIG. 2. Autoradiogram showing ^{125}I-IGF-I (lanes A and B), ^{125}I-MSA (lanes C and D), and ^{125}I-insulin (lanes E and F) receptor complexes crosslinked to chicken embryo fibroblasts and examined under reducing conditions (100 μM dithiothreitol). Fibroblasts were incubated in the absence and in the presence of unlabeled compounds as in Fig. 1.

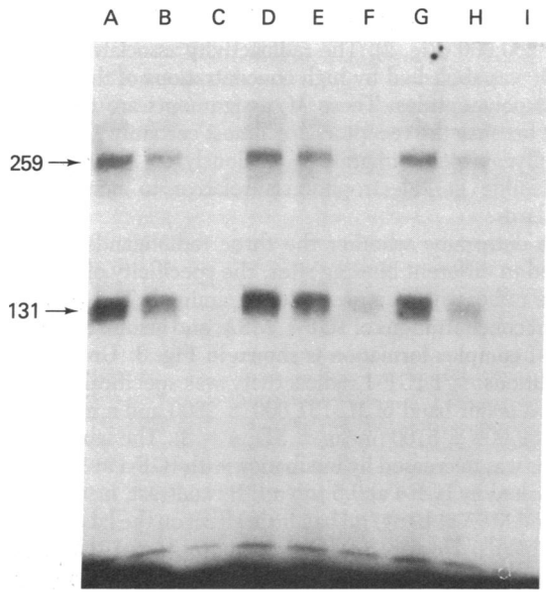

FIG. 3. Autoradiogram showing the specificity of ^{125}I-IGF-I–receptor complexes crosslinked to chicken embryo fibroblasts and examined under reducing conditions. Fibroblasts were incubated with ^{125}I-IGF-I in the absence (lane A) and in the presence of unlabeled IGF-I at 10 and 500 ng/ml (lanes B and C), unlabeled insulin at 10, 100, and 1000 ng/ml (lanes D, E, and F) and unlabeled MSA at 10, 100, and 1000 ng/ml (lanes G, H, and I).

MSA to the M_r 131,000 and 257,000 bands is consistent with the results of competitive binding studies (18).

^{125}I-Insulin was specifically crosslinked to a predominant band of M_r 126,000 ± 1200 and a minor band of M_r 252,000 ± 6900 (mean ± SD; n = 3). Unlabeled insulin decreased and finally abolished the radioactivity associated with these two bands in a dose-dependent manner (10 ng/ml to 1 μg/ml) (Fig.

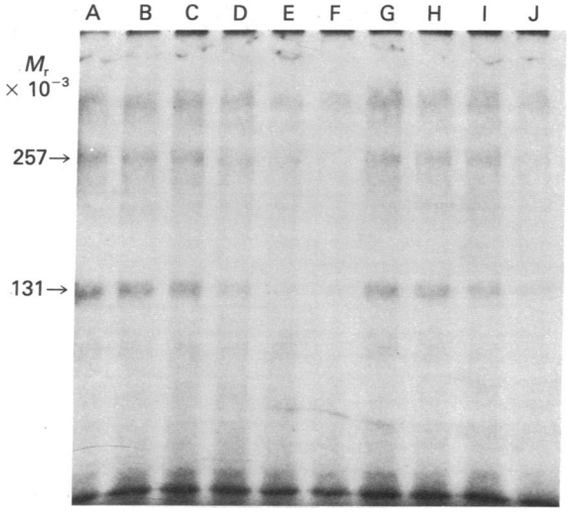

FIG. 4. Autoradiogram showing the specificity of ^{125}I-MSA–receptor complexes crosslinked to chicken embryo fibroblasts and examined under reducing conditions. Fibroblasts were incubated with ^{125}I-MSA in the absence (lane A) and in the presence of unlabeled MSA at 10, 30, 100, 300, and 1000 ng/ml (lanes B, C, D, E, and F) or insulin at 30, 100, 300, and 1000 ng/ml (lanes G, H, I, and J). In addition to the M_r 257,000 and 131,000 bands (arrows), radioactivity also was present in a band corresponding to M_r 300,000. Formation of this latter band was not inhibited by unlabeled MSA or insulin, suggesting that it represents nonspecific binding.

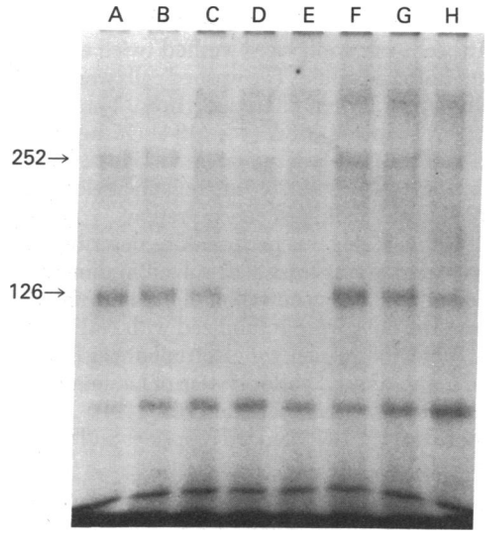

FIG. 5. Autoradiogram showing the specificity of ^{125}I-insulin–receptor complexes crosslinked to chicken embryo fibroblasts and examined under reducing conditions. Fibroblasts were incubated with ^{125}I-insulin in the absence (lane A) and in the presence of unlabeled insulin at 10, 100, 1000, and 10,000 ng/ml (lanes B, C, D, and E) or unlabeled MSA at 10, 100, and 1000 ng/ml (lanes F, G, and H).

5). Unlabeled MSA was less potent than insulin in decreasing the labeling of these bands. The radioactivity in the M_r 126,000 band was decreased to similar extents by insulin at 10 ng/ml and MSA at 100 ng/ml and by insulin at 100 ng/ml and MSA at 1 μg/ml, suggesting an approximate 10-fold difference in potency. Similar specificity of the chicken embryo fibroblast insulin receptor has been observed in competitive binding studies (18).

To determine whether the M_r ≈260,000 IGF receptor protein existed before exposure to chemical crosslinking agents, ^{125}I-MSA was crosslinked to its receptor with different concentrations of disuccinimidyl suberate. When the concentration of crosslinking reagent was increased from 0.01 to 0.5 mM, the ratio of radioactivity in the M_r 257,000 band compared to that in the M_r 131,000 band increased (Fig. 6 *Lower*). The radioactivity associated with both labeled bands was quantitated in slices of the gel (Fig. 6 *Upper*). The ratio of radioactivity in the M_r 257,000 and 131,000 bands increased from 0.24 to 1.15, approximately 5-fold, with increasing concentration of crosslinking reagent. This suggests that the M_r 257,000 complex may arise, at least in part, by crosslinking of the M_r 131,000 protein to itself or other membrane proteins.

DISCUSSION

The IGFs are a group of polypeptide hormones chemically related to insulin and thought to have arisen from an ancestral insulin gene (1, 2). Insulin and the IGFs have the same spectrum of biological activities *in vitro*, although the relative potencies of the two peptides vary for different functions (7). IGFs crossreact with insulin receptors; insulin crossreacts with some, but not all, IGF receptors (6, 10).

A coherent picture of the structure of the insulin receptor has emerged from recent studies using photoaffinity labeling (21, 22), affinity crosslinking (23, 24), biosynthetic labeling (25), and receptor purification (26). Specifically, the receptor appears to consist of subunits of M_r 130,000 and subunits of M_r 95,000 linked by disulfide bonds (22, 25, 27). The M_r 130,000 subunit is preferentially labeled by affinity labeling and appears to be the subunit that binds insulin; the M_r 95,000 nonbinding sub-

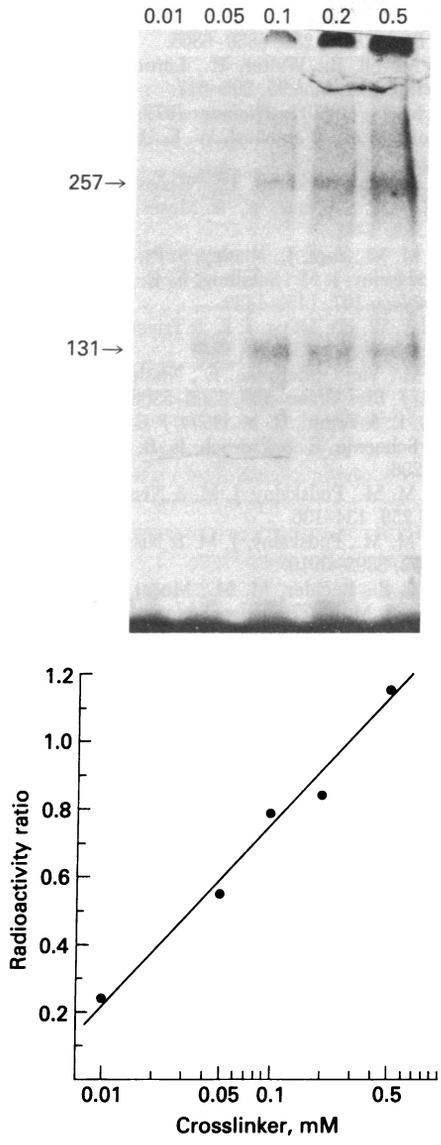

FIG. 6. (*Upper*) Effect of the concentration (shown as mM) of crosslinking reagent (disuccinimidyl suberate) on the formation of ^{125}I-MSA–receptor complexes in chicken embryo fibroblasts, and examined under reducing conditions. (*Lower*) Ratio of radioactivities, M_r 257,000/M_r 131,000, in slices of the gel shown in *Upper*.

unit is only weakly labeled after crosslinking with disuccinimidyl suberate (24, 27) but is strongly labeled when the insulin receptor of IM-9 cultured lymphocytes is biosynthetically labeled with [^{35}S]methionine and immunoprecipitated by antibodies to insulin receptors (25). Minor species of M_r ≈45,000 have been described that appear to result from partial proteolysis (28).

By contrast, when examined after covalent crosslinking with disuccinimidyl suberate, IGF receptors exhibit two different subunit structures (14). Type I IGF receptors do not interact with insulin and are composed of a M_r ≈260,000 protein that is not linked by disulfide bonds to other membrane proteins. This type of receptor has been identified with ^{125}I-MSA in rat liver membranes (14, 29), the BRL 3A2 rat liver cell line (14), rat adipocytes (29), rat embryo fibroblasts††, and chondrocytes from a rat chondrosarcoma††. The second IGF receptor structure, type II, initially demonstrated with ^{125}I-IGF-I in the BRL 3A2 rat liver cell line (14), consists predominantly of a M_r ≈130,000 protein that interacts with insulin and is linked by disulfide bonds to other membrane proteins to form high molecular weight (M_r >300,000) complexes under nonreducing conditions. IGF receptors with this structure have also been demonstrated with ^{125}I-IGF-I in human placental membranes (30, 31, ††) and cultured human fibroblasts††.

The present study demonstrates that ^{125}I-IGF-I and ^{125}I-MSA are crosslinked to type II receptors in chicken embryo fibroblasts. When ^{125}I-MSA and ^{125}I-IGF-I were crosslinked to chicken embryo fibroblasts, radioactivity appeared in one major and one minor high molecular weight complex (M_r >300,000). After disulfide reduction, the radioactivities of ^{125}I-MSA– and ^{125}I-IGF-I–receptor complexes appeared predominantly at M_r ≈130,000. Formation of these complexes was inhibited by unlabeled IGF-I, MSA, and insulin, in that order of potency. These results suggest that the structure of the receptor for ^{125}I-MSA is identical to that for ^{125}I-IGF-I and that the receptor for both ligands may be the same protein. A similar conclusion was reached from competitive binding studies; the specificity of inhibition of binding of ^{125}I-MSA and ^{125}I-IGF-I to chicken embryo fibroblasts was identical (12).

Under reducing conditions, radioactivity of ^{125}I-IGF-I and ^{125}I-MSA crosslinked to chicken embryo fibroblasts also appeared in a minor complex of M_r ≈260,000. With increasing concentration of crosslinking agent, the proportion of radioactivity in the M_r ≈260,000 region relative to that in the M_r ≈130,000 region increased approximately 5-fold. This suggests that the M_r ≈260,000 complex arises at least in part by crosslinking of the M_r ≈130,000 protein to other proteins, most probably an adjacent M_r 130,000 subunit. Although it cannot be excluded that a separate M_r ≈260,000 receptor exists, as in the type I IGF receptors, we consider this less likely for two reasons: (*i*) formation of the type I IGF receptor is not inhibited by insulin, whereas the M_r ≈260,000 complex observed in chicken embryo fibroblasts shows the same inhibition by insulin as the M_r ≈130,000 complex, and (*ii*) the M_r ≈260,000 band in type I IGF receptors has an apparent M_r of 220,000 before reduction, presumably the result of disulfide bonds maintaining a more compact structure, and does not aggregate to larger forms. We have not detected a M_r ≈220,000 band in chicken embryo fibroblasts in the absence of reduction under our experimental conditions. However, we cannot exclude the possibility that some of the radioactivity in the region of M_r ≈260,000 in reducing conditions represents radioactivity crosslinked to a type I IGF receptor.

Although previous results (14, 30, 31, ††) identified type I IGF receptors (insulin-insensitive) by using ^{125}I-MSA and type II IGF receptors (insulin-sensitive) by using ^{125}I-IGF-I, the present results indicate that, in chicken embryo fibroblasts, ^{125}I-MSA binds preferentially to a type II IGF receptor. Thus, the reactivity of ^{125}I-MSA is not limited to type I IGF receptors, although this radioligand may bind preferentially to type I receptors when both types of IGF receptors are present in the same tissue.

The chicken embryo fibroblast insulin receptor is similar in size to the IGF receptor under both reducing and nonreducing conditions. The slightly smaller size of the insulin–receptor complex results, at least in part, from differences in the size of the bound radioligand: ^{125}I-insulin A chain, M_r = 2200; ^{125}I-IGF-I, M_r ≈7500; ^{125}I-MSA, M_r ≈ 8700). Insulin–receptor and IGF–receptor complexes may be differentiated by the specificity of inhibition by insulin, MSA, and IGF-I. IGF-I most potently inhibits formation of the ^{125}I-IGF-I–receptor complex;

†† Kasuga, M., Van Obberghen, E., Nissley, S. P. & Rechler, M. M. (1981) Program of the 63rd Annual Meeting of the Endocrine Society, Cincinnati, OH, June 17–19, 1981, Abstr. 617, p. 237.

insulin most potently inhibits formation of the ^{125}I-insulin–receptor complex. The possibility that the ^{125}I-insulin and ^{125}I-IGF-I binding sites coexist on the same M_r 130,000 protein cannot be excluded. Although we have not identified M_r 95,000 components for the chicken embryo fibroblast IGF receptor, by analogy with the proposed structure of the insulin receptor (32) and from the presence of higher molecular weight forms under nonreducing conditions, an IGF receptor structure consisting of M_r 130,000 and M_r 95,000 proteins is consistent with our results. The presence of M_r 95,000 subunits has been inferred for the type II IGF receptor in human placental membranes (31).

In contrast to other tissues, which appear to possess both types of IGF receptor structures, the chicken embryo fibroblast appears to possess a single type of IGF receptor that interacts with all ^{125}I-labeled IGF peptides. Because these peptides exert mitogenic effects with the same relative potencies and at similar concentrations as for their effects in binding experiments, we believe that the IGF receptor whose subunit structure has been described in this paper is a functional receptor that mediates the mitogenic effects of the IGFs in chicken embryo fibroblasts.

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