Chicken and Xenopus Mannose 6-Phosphate Receptors Fail to Bind Insulin-like Growth Factor II*

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The recent demonstration that a single mammalian receptor protein binds both mannose 6-phosphate (Man-6-P) and insulin-like growth factor II (IGF-II) with high affinity has suggested a multifunctional physiological role for this receptor, possibly including signal transduction. In order to better understand the functions of this receptor, we have investigated the properties of Man-6-P receptors from non-mammalian species. Receptors were affinity-purified from Triton X-100 extracts of total membranes from Xenopus and chicken liver as well as rat placenta using pentamannosyl 6-phosphate-Sepharose. The Man-6-P receptor was adsorbed to the pentamannosyl 6-phosphate-Sepharose and specifically eluted by Man-6-P in all three species, as evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining. When the purified receptors from these three species were cross-linked to 125I-IGF-II with disuccinimidyl suberate, only receptors isolated from rat membranes were affinity-labeled. To further evaluate the properties of these Man-6-P receptors, binding of 125I-rat-IGF-II and 125I-chicken Tyr-Gly-Thr-Ala-IGF-II to purified receptors from Xenopus, chicken, and rat was evaluated by polyethylene glycol precipitation. Only the rat Man-6-P receptor exhibited detectable binding of 125I-IGF-II. These data suggest that the emergence of a high affinity IGF-II binding site on the Man-6-P receptor occurred in evolution after the divergence of mammals from other vertebrates. Thus, the biological actions of IGF-II in chickens and frogs appear to be initiated by the type I IGF receptor.

The insulin-like growth factors I and II (IGF-I and -II) are widespread among animals, yet the physiologic roles and mechanisms of action of these hormones are incompletely understood. The synthesis and release of IGF-I into the circulation has been demonstrated to be regulated by growth hormone (1), and IGF-I induces growth when administered directly (1). Similarly, IGF-II has been implicated in a number of physiologic effects, including stimulation of thymidine incorporation (2–5), RNA and protein synthesis (6), Ca²⁺ influx (2), glucose (6–7), and amino acid (6) uptake, glucose incorporation into glycogen (8), glucose oxidation (4), protein phosphorylation (9), and cell proliferation (3) and differentiation (10). IGF-II has also been reported to inhibit protein degradation (6) and change intracellular pH (11). Biological responses of IGF-I and IGF-II have been shown to be mediated by a type-I IGF receptor which is structurally very similar to the insulin receptor, a heterotetrameric tyrosine kinase composed of two α and two β subunits (1). Another receptor for IGF-II, the type II IGF receptor, bears no resemblance to these signaling receptors. It consists of a single polypeptide chain with one transmembrane segment, a short cytoplasmic domain, and no protein kinase activity (12–18). Furthermore, this IGF-II receptor has been shown to be identical to the cation independent Man-6-P receptor as demonstrated by sequence analysis (12–16) and binding studies (12).

A number of experiments have been designed to elucidate through which receptor or receptors the biological activities of IGF-II are mediated. Some of these studies indicate that IGF-II effects are mediated through the insulin or IGF-I receptor, either by analysis of dose-response relationships or by the use of antibodies which block binding of peptide ligands to insulin (4) and IGF-I (6) receptors. Other results have been interpreted to suggest that some biological effects may be mediated through the IGF-II/Man-6-P receptor (7–9, 11). Some of these effects of IGF-II are mimicked by anti-IGF-II/Man-6-P receptor antiserum (2, 8). However, two preparations of anti-receptor antibodies which block ligand binding to the IGF-II/Man-6-P receptor did not block biological responses (19, 20). The signaling potential of the IGF-II/Man-6-P receptor thus remains controversial.

The presence of IGFs has been reported in chickens (21), reptiles, amphibians, and fish (22) where their presence has been assayed by radioimmunoassay or the ability to compete with the mammalian peptide for binding to mammalian IGF-II/Man-6-P receptor. Furthermore, mammalian IGFs have been shown to stimulate DNA synthesis, nutrient transport, and cell multiplication in chick embryo fibroblasts (3, 23) and in cultured chick myotubes (6). These peptides also act to initiate differentiation in chick myoblasts (9). Other processes affected by IGFs include DNA, RNA, and protein synthesis in chick chondrocyte cultures (24) and glycogen, RNA, and protein synthesis in chick liver cells (25). These data strongly support the hypothesis that the avian and mammalian IGFs have been highly conserved during evolution in both function and structure. The partial amino acid sequences of the chicken IGFs further support this conclusion (21). However, studies on binding and affinity cross-linking of 125I-IGF-II to cell membranes have indicated that chickens possess an IGF receptor which resembles the mammalian IGF-I receptor and binds both ligands with near equal affinity (3, 26). No labeling of a type II IGF receptor was detected in such experiments. In this study, we have addressed this issue directly by assessing the ability of affinity purified Man-6-P receptors from...
rat, chicken, and *Xenopus* to bind with rat and chicken 125I-IGF-II.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rat IGF-II was purified from BRL-3A cell-conditioned medium as described (27). Chicken Tyr-Gly-Thr-Ala variant form of IGF-II was a gift from Dr. F. J. Ballard, Commonwealth Scientific and Industrial Research Organization, Adelaide, South Australia. Purified IGF-II was radiiodinated using Enzymobeads (Bio-Rad) to a specific activity of 40–250 Ci/g. Pentamannosyl δ-phosphate (PMP), prepared from the native Y-2448 δ-phosphomannan from *Hapalotrichia holetti*, was a gift of Dr. M. E. Slodki, Midwest Area Northern Regional Research Center, Peoria, IL. PMP-Sepharose was prepared as described (28). Nitrocellulose filters with a 45-μm pore size were obtained from the Millipore Corporation. All other chemicals were reagent grade or better.

**Preparation of Membranes from Rat Placenta, Chicken Liver, and Frog Liver**—A microsomal membrane fraction was isolated from rat placentas taken at day 19 of gestation as described previously (29), and chicken and *Xenopus* liver membranes were prepared by the same technique. Membrane extracts (5 mg protein/ml) were prepared by incubation with 50 mM Hepes, 0.15 M NaCl, 1% Triton X-100, 5 mM β-glycerophosphate, pH 7.4, plus protease inhibitors by gentle mixing for 1 h at 3 °C. The protease inhibitors were leupeptin, antipain, and benzamidine at concentrations of 10 μg/ml each, 20 μg/ml aprotinin, 12.5 μg/ml chymostatin, and 1 mM phenylmethylsulfonyl fluoride. The mixture was centrifuged for 10 min at 15,000 × g in a microcentrifuge and the supernatant fraction was used as the extract of whole membranes was incubated with PMP-Sepharose overnight, and then this material was loaded onto a PMP-Sepharose column by affinity cross-linking. Triton X-100 extracts were prewashed in a high salt buffer as described under "Experimental Procedures." Fractions were eluted with 1 ml of buffer alone (not shown), buffer containing 5 mM mannose 1-phosphate (lanes 1, 4, and 7), buffer containing 5 mM glucose 6-phosphate (lanes 2, 5, and 8), or buffer containing 5 mM Man-6-P (lanes 3, 6, and 9). One aliquot of 0.1 ml of each fraction was then analyzed by SDS-PAGE followed by silver staining (panel B), whereas another aliquot (50 μl) was analyzed by cross-linking with diuccinimidyldimethylsulfate to 4 nM 125I-IGF-II, followed by SDS-PAGE and autoradiography (panel A). The apparent Mr of the receptor band in the cross-linking study is increased to 260,000 due to the added molecular mass of the cross-linked IGF-II.

**Affinity Cross-linking of 125I-IGF-II to Purified Receptors**—Following purification with PMP-Sepharose, receptors from 100 μg of membrane protein were suspended to a volume of 0.2 ml with Krebs Ringer phosphate buffer containing 1% bovine serum albumin plus 4 nM 125I-IGF-II with or without 400 nM unlabeled IGF-II. These mixtures were then incubated for 4 h at 23 °C. The samples were subjected to affinity cross-linking with diuccinimidyldimethylsulfate as described before (31).

**Affinity Cross-linking Method**—The studies of ligand binding to receptor were performed as described previously (29). Briefly, 0.1 ml of a stock solution of 4 nM 125I-IGF-II and unlabeled IGF-II at 0, 10, 40, 100, 400, and 1000 nM was combined with an equal volume of a solution of purified receptors at 4 μg of protein. Following incubation for 1 h at 3 °C, 0.5 ml of 0.9 mg/ml bovine γ-globulins in 0.1 M sodium phosphate buffer, pH 7.4, and 0.5% polyethylene glycol were added to each sample, the sample mixed, and incubated at 0 °C for 15 min. This mixture was then filtered through nitrocellulose filters which were blocked by incubation in Krebs Ringer phosphate buffer containing 1% bovine serum albumin, the filters washed three times with 8% polyethylene glycol, and the γ-radiation from the filters measured.

**RESULTS AND DISCUSSION**

In the first experimental protocol (Fig. 1), PMP-Sepharose was used to affinity-purify Man-6-P receptors from membrane extracts of rat placenta (lanes 1–3), chicken liver (lanes 4–6), and *Xenopus laevis* liver (lanes 7–9). A Triton X-100 extract of whole membranes was incubated with PMP-Sepharose overnight, and then this material was loaded onto a column and the fractions eluted by buffer alone (not shown), mannose 1-phosphate (lanes 1, 4, and 7), glucose 6-phosphate (lanes 2, 5, and 8), and Man-6-P (lanes 3, 6, and 9). From each fraction 1 aliquot was electrophoresed (SDS-PAGE) and then silver-stained (panel B). A duplicate sample was incubated with 125I-IGF-II and the cross-linking agent diuccinimidyldimethylsulfate to 4 nM 125I-IGF-II, followed by SDS-PAGE and autoradiography (panel A). The apparent Mr of the receptor band in the cross-linking study is increased to 260,000 due to the added molecular mass of the cross-linked IGF-II.

![Fig. 1. Analysis of fractions eluted from a PMP-Sepharose column by affinity cross-linking.](image-url)
the presence of IGFs has been demonstrated, and in the Xenopus three species described in Fig. 1. Although rat 125I-IGF-II binds significantly to rat receptor by a saturable process, no the experimental conditions described here. In these species, it is possible that IGF-II and its receptor have diverged in evolution such that ligand-receptor interactions are species-specific. Direct binding studies using both rat and chicken IGF-II using the purified Man-6-P receptors were performed to definitively test this hypothesis. In Fig. 2 (panel A) is shown the competition by excess unlabeled rat IGF-II of rat IGF-II binding to Man-6-P receptors purified from the three species described in Fig. 1. Although rat 125I-IGF-II binds significantly to rat receptor by a saturable process, no binding to the chicken or Xenopus receptor by this ligand is observed. This experiment was repeated (Fig. 2, panel B) using 125I-chicken IGF-II as ligand with similar results: neither chicken nor Xenopus Man-6-P receptors bind chicken 125I-IGF-II significantly, whereas the rat receptors bind this ligand with high affinity.

The data presented above demonstrate that chicken and Xenopus liver cells contain Man-6-P receptors but that they lack the ability to bind IGF-II with measurable affinity under the experimental conditions described here. In these species, the presence of IGFs has been demonstrated, and in the chicken, IGF-II has been shown to cause a number of physiologic responses, many similar to those documented in mammals. Importantly, the chicken Man-6-P receptor fails to bind chicken 125I-IGF-II with high affinity, whereas the rat receptor binds this ligand with high affinity. It has been demonstrated previously that chickens possess a type I IGF receptor tyrosine kinase which binds IGF-I and -II with near equal affinity (9). It thus seems likely that both IGF-I and IGF-II mediate biological actions by interacting with this type I IGF receptor in chickens.

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Note Added in Proof—While this manuscript was undergoing review, a paper was published (Canfield, W. M., and Kornfeld, S. (1989) J. Biol. Chem. 264, 7100–7103) which also reported that the chicken Man-6-P receptor failed to bind IGF-II with high affinity.

REFERENCES

A.

B.

Fig. 2. Analysis of the binding of 125I-rat and chicken IGF-II to purified Man-6-P receptors. Receptors purified on PMP-Sepharose (4 mg of protein) were incubated in a total volume of 0.2 ml with 2 nM 125I-IGF-II from rat (panel A) or chicken (panel B) containing 0.5, 20, 50, 200, or 500 nM unlabeled rat IGF-II. Following a 1-h incubation at 3°C, samples were polyethylene glycol-precipitated and the γ-radiation from the filters measured.

B.

Non-mammalian Man-6-P Receptors Fail to Bind IGF-II

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