Developmental Expression of the Tissue Insulin-like Growth Factor II/Man nose 6-Phosphate Receptor in the Rat

MEASUREMENT BY QUANTITATIVE IMMUNOBLOTTING*

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Mark M. Sklar, Wieland Kiess†, Cheryl L. Thomas, and S. Peter Nissley§
From the Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

We used quantitative immunoblotting to measure the total tissue insulin-like growth factor II/man nose 6-phosphate (IGF-II/Man-6-P) receptor in the rat. Whole embryos (6–15 days of gestation) and tissues from 16- and 20-day-old fetal and 5-, 10-, 20-, and 40-day-old postnatal rats were placed in liquid nitrogen, extracted with 2% Triton X-100, and boiled in 2% sodium dodecyl sulfate. The extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis together with aliquots of a highly purified rat IGF-II/Man-6-P receptor standard. The protein bands were transferred from the gel to nitrocellulose sheets by electroelution. The nitrocellulose sheets were incubated with a specific IGF-II/Man-6-P receptor antiserum (36337). The immunoblots were developed with 

insulin (1). IGF-I secretion is dependent on pituitary growth hormone, and IGF-I is a mediator of the anabolic effects of growth hormone (2–4). The precise function of IGF-II, however, has not been elucidated. Earlier experiments in the rat, demonstrating that serum IGF-II and tissue IGF-II mRNA are present at high levels prenatally and decline postnata

ly, have led to speculation that this growth factor may have an important role in fetal growth and development (5–10). There are receptors for each of the growth factors in many different cell types. The IGF-I receptor binds IGF-I, IGF-II, and insulin in order of decreasing affinity (11). The IGF-II receptor binds IGF-II most avidly; and although it binds IGF-I, the IGF-II receptor does not recognize insulin. Structurally, the IGF-I receptor resembles the insulin receptor (12). Both the IGF-I and insulin receptors are tetramers with two α-subunits which bind ligand and two β-subunits. The β-subunits have intrinsic tyrosine kinase activity. The receptor for IGF-II, whose structure was recently determined by cloning and sequencing the receptor cDNA (13–15), bears no resemblance to the IGF-I or insulin receptor. It consists predominantly of an extracellular domain with 15 repeating units of approximately 150 amino acids each. The short cytoplasmic domain does not exhibit homology to known tyrosine kinases. Interestingly, the amino acid sequence of the IGF-II receptor was found to be identical to the cation-independent man nose 6-phosphate receptor (13–15). Identity of the two receptors was confirmed with biochemical and immunological experiments (16–20). Thus, the IGF-II/Man-6-P receptor is a multifunctional protein which is involved in targeting lysosomal enzymes bearing the man nose 6-phosphate recognition marker to lysosomes (21–23) and in binding IGF-II.

We have recently reported that the IGF-II/Man-6-P receptor is developmentally regulated in rat serum, with high levels in fetal serum declining dramatically between 20 and 40 days after birth (24). In this study, using a technique of quantitative immunoblotting, we measured the receptor in solubilized whole tissue extracts from rats ranging in age from 6 days before to 40 days after birth to determine if there is a development pattern for IGF-II/Man-6-P receptor expression similar to that seen in rat serum. Whole embryos 6–15 days after conception were also assayed for the IGF-II/Man-6-P receptor.

MATERIALS AND METHODS

Rat Tissues—Timed pregnant Sprague-Dawley rats were purchased from Zivic-Miller Laboratories (Zelienople, PA). Animals were killed by CO₂ narcosis at 6, 8, 10, 12, 14, 15, 16, and 20 days of gestation. Sprague-Dawley rats were killed 5, 10, 20, and 40 days after birth. Fetuses were separated from placental tissues. Sixteen- and 20-day-old fetal and postnatal rats were dissected to obtain the following tissues: brain, heart, intestine, kidney, liver, limb (in fetal animals), lung muscle (in adult animals), pancreas, placenta, and spleen. Tis—

Insulin-like growth factors (IGF) I and II are mitogenic polypeptides whose amino acid sequence shows homology to insulin (1). IGF-I secretion is dependent on pituitary growth hormone, and IGF-I is a mediator of the anabolic effects of growth hormone (2–4). The precise function of IGF-II, however, has not been elucidated. Earlier experiments in the rat, demonstrating that serum IGF-II and tissue IGF-II mRNA are present at high levels prenatally and decline postnatally, have led to speculation that this growth factor may have an important role in fetal growth and development (5–10). There are receptors for each of the growth factors in many different cell types. The IGF-I receptor binds IGF-I, IGF-II, and insulin in order of decreasing affinity (11). The IGF-II receptor binds IGF-II most avidly; and although it binds IGF-I, the IGF-II receptor does not recognize insulin. Structurally, the IGF-I receptor resembles the insulin receptor (12). Both the IGF-I and insulin receptors are tetramers with two α-subunits which bind ligand and two β-subunits. The β-subunits have intrinsic tyrosine kinase activity. The receptor for IGF-II, whose structure was recently determined by cloning and sequencing the receptor cDNA (13–15), bears no resemblance to the IGF-I or insulin receptor. It consists predominantly of an extracellular domain with 15 repeating units of approximately 150 amino acids each. The short cytoplasmic domain does not exhibit homology to known tyrosine kinases. Interestingly, the amino acid sequence of the IGF-II receptor was found to be identical to the cation-independent man nose 6-phosphate receptor (13–15). Identity of the two receptors was confirmed with biochemical and immunological experiments (16–20). Thus, the IGF-II/Man-6-P receptor is a multifunctional protein which is involved in targeting lysosomal enzymes bearing the man nose 6-phosphate recognition marker to lysosomes (21–23) and in binding IGF-II.

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sues were diced, washed in PBS, and immediately frozen in liquid nitrogen. Embryos under 16 days of age were frozen whole in liquid nitrogen.

**Tissue Extraction**—Approximately 5 mg of thawed tissue was placed in 1.5-ml polypropylene tubes and weighed. One ml of 20 mM Tris, 2% Triton X-100 was added, and the tissue was homogenized for 2 min with a hand-held microcurette (RPI catalog number 9922; Research Products International, Mount Prospect, IL). Laemmli buffer (25) containing 2% SDS (0.25 ml was added; each tube was boiled for 5 min and then incubated at 4 °C overnight. Aliquots of extract were stored at −70 °C.

**Immunoblotting**—Thawed extracts were boiled for 1 min and centrifuged for 1 min at 13,000 rpm. An aliquot of the supernatant equivalent to 200 µg of tissue together with 620, 310, 155, and 77.5 ng of myosin, 200,000 (Amersham Corp.) were transferred by electroelution onto nitrocellulose paper (Schleicher & Schuell), and immunoblotting was performed with IGF-II/Man-6-P receptor antiserum (3837) and F1-antigen A as described (28). Nitrocellulose sheets were then stained using an immunoperoxidase staining kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Nitrocellulose sheets were incubated for 1 h at room temperature in 10 ml of PBS containing 3 drops of goat anti-rabbit biotinylated IgG. Sheets were washed four times over a 20-min interval and incubated with avidin, and 3 drops of avidin, and 3 drops of biotinylated horseradish peroxidase. Blots were washed again four times over a 20-min period. Peroxidase substrate (4-chloro-1-naphthol (Sigma)) at 3 mg/ml in 10 ml of methanol and 60 µl of 33% hydrogen peroxide in 50 ml of 50 mM Tris, 200 mM NaCl, pH 7.4, were poured on the nitrocellulose sheet, and the reaction was allowed to proceed until the desired amount of staining was attained. The reaction was quenched with excess PBS. After drying overnight, nitrocellulose sheets were autoradiographed with Kodak X-AR film and Du Pont-New England Nuclear enhancing screens. Areas were cut from each Western blot and subjected to autoradiography with Kodak X-AR film and Du Pont-New England Nuclear enhancing screens (Cricket graph, Cricket Software, Philadelphia, PA). The equation describing the curve permitted calculation of IGF-II/Man-6-P receptor content in each of the tissues assayed.

**Protein Determination**—Tissue extracts were centrifuged for 1 min at 13,000 rpm. The protein content in an aliquot of supernatant was determined using BCA protein assay reagent (Pierce Chemical Co). Dilutions of bovine serum albumin were used as standards.

**Statistics**—Student’s t test was performed using the computer program StatWorks (Cricket Software) to determine whether differences in IGF-II/Man-6-P receptor content between fetal and postnatal tissues were statistically significant.

### RESULTS

**Adequacy of Tissue Extraction**—Initial extraction was carried out as described under “Materials and Methods.” To assess if extraction of the IGF-II/Man-6-P receptor was complete, extracts of representative tissues from animals of varying ages (liver from 18-day-old fetal rats; intestine, heart, brain, liver, muscle, and kidney from 5- and 40-day-old postnatal rats) were centrifuged at 13,000 rpm for 3 min in a microcentrifuge, and the supernatant was decanted. The pellet material was washed three times in water; resuspended in 1% CHAPS (Sigma), 100 mM NaCl; and boiled for 1 min. After an overnight incubation at 4 °C, the suspension was centrifuged; and the supernatant and the initial extract supernatant were subjected to SDS-PAGE, electroelution, and autoradiography as described under “Materials and Methods.” We could not detect the IGF-II/Man-6-P receptor in the CHAPS-solubilized pellet from fetal or postnatal tissues (data not shown).

To determine if use of protease inhibitors would increase the yield of receptor from extracted material, tissues of 20-day-old fetal and 20-day-old postnatal rats and whole embryos were extracted as described under “Materials and Methods,” but with the addition of antipain at 125 µg/ml (Sigma) and phenylmethylsulfonyl fluoride at 5 mg/ml (Sigma) to the 20 mM Tris, 2% Triton X-100 extraction medium. Extracts were subjected to SDS-PAGE, electroelution, and immunoprecipitation as described. Fig. 4, which compares results of extraction of whole embryos in the absence (line 1) or the presence (line 2) of phenylmethylsulfonyl fluoride, reveals that the quantity of the IGF-II/Man-6-P receptor is not greater in the extract with the protease inhibitors. Also, receptor recovery does not appear to be enhanced by addition of protease inhibitors to 20-day-old fetal or 20-day-old postnatal tissues (data not shown).

**Immunoprecipitation**—As detailed under “Materials and Methods,” aliquots of highly purified IGF-II/Man-6-P receptor standard were subjected to SDS-PAGE and electroelution along with each group of tissue extracts (Fig. 1). Receptor-antibody complexes on the nitrocellulose sheets were doubly labeled with radiiodinated protein A and an immunoperoxidase label. This enabled us to visualize areas on the nitrocellulose sheets containing the IGF-II/Man-6-P receptor. Fig 1 (A and B) illustrate a peroxidase-stained Western blot and its autoradiogram, respectively. The dyed areas were cut from the nitrocellulose sheets, and the radioactivity was measured in a γ-counter. A standard curve relating known protein quantities of purified receptor to radioactivity was generated for each gel (Fig. 2). We found our technique of dual labeling receptor-antibody complexes about 30 times more sensitive for detecting γ-emission than the AMBIS Scanning System (Automated Microbiology Systems, Inc., San Diego, CA) (data not shown).

**IGF-II/Man-6-P Receptor Is Developmentally Regulated**—We found that the concentration of the IGF-II/Man-6-P receptor expressed as percent of total protein in the tissue extract was highest in the 16- and 20-day-old fetal tissues and declined dramatically by day 40 of postnatal development.

![Fig. 1. Immunoprecipitation of tissue IGF-II/Man-6-P receptor](image-url)
lanes containing the purified IGF-II/Man-6-P receptor (Fig. 1) were cut from each blot as described under "Materials and Methods." The receptor: standard curve. The radioactivity of each strip was measured by gamma-counting. The content of receptor varied among the organs studied. At 16 days of fetal development, following heart in decreasing order of IGF-II/Man-6-P receptor concentration, were placenta (1.0%), lung (0.7%), intestine (0.7%), muscle (0.5%), kidney (0.5%), liver (0.3%) and brain (0.1%). Pancreas and spleen could not be isolated at 16 days of fetal development. In general, the IGF-II/Man-6-P receptor concentration fell dramatically late in gestation and/or after birth in all tissues, except for brain, pancreas, and spleen. Examination of the slope of the receptor concentration curve revealed that the greatest decrease for most tissues (heart, placenta, lung, and intestine) occurred between days 16 and 20 of fetal development (Fig. 4). In muscle and intestine, there was a small increase in receptor concentration postnatally at days 5 and 10, respectively.

The differences between IGF-II/Man-6-P receptor concentration in fetal (16 and 20 days) and postnatal (20 and 40 days) tissues were significant for all tissues studied (Table I). Although receptor content was reduced to approximately 0.05% total protein in 40-day-old postnatal tissues, the IGF-II/Man-6-P receptor was still easily detectable by immunoblotting (Fig. 3). We were able to isolate whole embryos as early as 6 days after conception. The IGF-II/Man-6-P receptor was easily detectable in 6-day-old embryos (Fig. 4).

Variation in Size of IGF-II/Man-6-P Receptor—The molecular weight of the IGF-II/Man-6-P receptor varied among the organs studied (Fig. 5). The differences became most pronounced in organs from older animals. By 20 days of gestation, liver appears to have a higher molecular weight receptor than the other tissues on the immunoblot. By 20 days after birth, the size of the IGF-II/Man-6-P receptor segregates the organs into two groups. The receptors in liver, heart, and lung have an apparent M, of approximately 230,000 whereas those in brain, intestine, kidney, muscle, pancreas, and spleen have an apparent M, of 220,000. In several tissues, the molecular weight of the IGF-II/Man-6-P receptor changes over time (Fig. 3). The receptors in brain and intestine decrease in size, whereas those in liver increase in size during postnatal development.

DISCUSSION

We have recently demonstrated (24) that the IGF-II/Man-6-P receptor is developmentally regulated in rat serum, with the highest levels in fetal and neonatal life which then decline dramatically between days 20 and 40 of postnatal development. The data presented in this paper indicate that for the most part, the ontogeny of this receptor in tissues parallels that in serum. The highest concentrations of the IGF-II/Man-6-P receptor was found in 16- and 20-day-old fetal tissues, with a marked decline during postnatal life. Although the IGF-II/Man-6-P receptor concentration in tissues fell earlier (between 10 and 20 days post partum) than that in serum (between 20 and 40 days) even in tissues with the lowest concentrations, the IGF-II/Man-6-P receptor was at least 5-fold greater than the highest concentration in serum. (Assuming that 1 ml of serum is equivalent to 1 g of tissue, the highest concentration of the IGF-II/Man-6-P receptor observed in serum was 5 µg/g, whereas the lowest concentration of the receptor in tissues was 25 µg/g.) Alexandrides et al. (29) recently reported that the IGF-II/Man-6-P receptor was much higher in fetal skeletal muscle than in postnatal...
Developmental Pattern of Tissue IGF-II/Man-6-P Receptor. Values from immunoinquantitation of whole tissue extracts for the IGF-II/Man-6-P receptor are expressed as percent of protein in the tissue extract. The left and upper right panels show data from fetal rats of 16 (−6), 20 (−2), 5, 10, 20, and 40 days old. The lower right panel shows data for whole embryos at 6 (−16), 8 (−14), 10 (−12), 12 (−8), 14 (−8), and 15 (−7) days of gestation. Line 1 refers to the first group of embryos tested. Line 2 refers to a repetition of the experiment using a different group of embryos. Protease inhibitors, antipain and phenylmethylsulfonyl fluoride, were present during the preparation of the second group of embryo extracts.

Table I
Comparison of the levels of the IGF-II/Man-6-P receptor in fetal and postnatal tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% of extract</th>
<th>p value</th>
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<tbody>
<tr>
<td></td>
<td>Fetal (−6)</td>
<td>Postnatal</td>
</tr>
<tr>
<td>Brain</td>
<td>0.11 ± 0.006</td>
<td>0.04 ± 0.006</td>
</tr>
<tr>
<td>Heart</td>
<td>1.03 ± 0.590</td>
<td>0.07 ± 0.017</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.49 ± 0.212</td>
<td>0.04 ± 0.017</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.45 ± 0.095</td>
<td>0.06 ± 0.025</td>
</tr>
<tr>
<td>Liver</td>
<td>0.31 ± 0.046</td>
<td>0.04 ± 0.015</td>
</tr>
<tr>
<td>Lung</td>
<td>0.51 ± 0.230</td>
<td>0.10 ± 0.032</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.52 ± 0.049</td>
<td>0.04 ± 0.020 &lt;0.001</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.16 ± 0.007</td>
<td>0.10 ± 0.020</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.14 ± 0.007</td>
<td>0.04 ± 0.015</td>
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The developmental pattern of the IGF-II/Man-6-P receptor parallels that of IGF-II. Concentrations of IGF-II measured in fetal rat serum are 20–100-fold higher than in adult serum and decrease after birth (5). In tissues, there is a high abundance of IGF-II messenger RNA in fetal compared with adult rats; and for most organs examined, levels of message reach a nadir by 10–20 days post partum (6). Similarly to the IGF-II receptor concentration, IGF-II mRNA varied among the tissues studied. However, there appears to be no correlation between the relative content of the IGF-II receptor in different tissues (heart > placenta > lung = intestine > liver > brain) and IGF-II mRNA in most 16-day-old fetal tissues. Interestingly, IGF-II mRNA expression, like the concentration of IGF-II/Man-6-P receptor protein, was low in neural tissues at the earliest developmental stages examined (6).

Our finding that the size of the IGF-II/Man-6-P receptor segregates into two groups in older animals, with heart, liver, and lung having an apparent Mr, of 230,000 and brain, intestine, kidney, muscle, and pancreas having a Mr of 220,000, is in accordance with previous reports (31, 32). Our findings differ from those of Taylor et al. (32) in the types of tissues assayed and their observation that lung was in the group with a lower molecular weight receptor. The size of the IGF-II/Man-6-P receptor also appears to change during development in certain organs. Receptor size decreases in brain and intestine and increases in liver between 20 days of gestation and postnatal age.
Developmental Expression of IGF-II/Man-6-P Receptor

20 days post partum. These alterations may be due to differences in glycosylation (31).

High levels of the IGF-II/Man-6-P receptor during embryonic and fetal life suggests a role in tissue remodeling. As more lysosomal enzymes are needed and produced for the remodeling of fetal tissue, perhaps increased quantities of the IGF-II/Man-6-P receptor are required to target these enzymes into lysosomes. In heart, where the IGF-II/Man-6-P receptor concentration is nearly 2% of total protein in the extract, it is possible that the receptor is involved in targeting enzymes involved in digestion of extracellular matrix. Studies on chick heart, for example, reveal that the lysosomal enzyme hyaluronidase is elevated during a period when the extracellular matrix (containing about 50% hyaluronate) between myocardium and endocardium is degraded as mesenchymal cushion cells invade and form valves and septa. Hyaluronidase falls precipitously between 16 and 20 days of fetal life after hyaluronate falls between 16 and 20 days to 30% of its initial level (33-35). Studies (36, 37) on embryonic chick cornea and regenerating new limbs also demonstrate increased concentrations of hyaluronidase as the extracellular matrix is degraded, followed by a fall in this enzyme in more differentiated tissues. Therefore, this is the possibility of a correlation between concentrations of the IGF-II/Man-6-P receptor and its lysosomal enzyme ligands in tissues undergoing remodeling.

The high concentrations of both the IGF-II/Man-6-P receptor and IGF-II during fetal life raise interesting questions about the functional interaction of a growth factor with a receptor which binds approximately 50 lysosomal enzymes. We have recently shown (38) that IGF-II inhibits uptake of the lysosomal enzyme β-galactosidase in C6 glial cells and BRL-3A cells in culture. The inhibition of cellular uptake is explained in part by the ability of IGF-II to inhibit binding of β-galactosidase to the IGF-II/Man-6-P receptor. Perhaps such interactions modulate the extracellular concentration of lysosomal enzymes in tissues that make IGF-II. For example, it is possible that high extracellular concentrations of IGF-II could potentiate remodeling of extracellular matrix material by inhibiting uptake of secreted lysosomal enzymes. In addition, since the receptor and both its ligands pass through the Golgi apparatus following their synthesis, it is possible that endogenously made IGF-II could bind to intracellular receptors and inhibit delivery of enzymes to lysosomes, resulting in delivery of more lysosomal enzymes outside the cell.

Since there are reports that suggest signaling of responses to IGF-II via the IGF-II/Man-6-P receptor, it is also possible that the coincidence of high levels of IGF-II and its receptor in fetal tissue points to a signaling function for the receptor apart from the targeting of lysosomal enzymes to lysosomes. Thus, the IGF-II/Man-6-P receptor has been reported to mediate stimulation of Ca²⁺ influx and DNA synthesis in 3T3 mouse fibroblasts (39, 40), inositol triphosphate and diacylglycerol production by basolateral membranes of canine proximal tubules (41), glycosen synthesis in a human hepatoma cell line (HepG2) (42), and clonal growth of an erythroleukemia cell line (K562) (43).

We conclude that the IGF-II/Man-6-P receptor is developmentally regulated and variably expressed in different tissues in the rat. The relatively high levels of the receptor in fetal tissues suggests that the receptor may play an important role in fetal growth and development.

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