Mannose 6-phosphate/insulin-like growth factor II-binding proteins in human serum and urine

Their relation to the mannose 6-phosphate/insulin-like growth factor II receptor

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Human serum and urine contain polypeptides which bind mannose 6-phosphate (M6P) and insulin-like growth factor II (IGF II) and crossreact with antibodies against the M6P/IGF II receptor. These polypeptides are considered to be fragments of the M6P/IGF II receptor. The major M_r approx. 205 000 fragment in serum and urine is about 10 kDa smaller in size than the membrane-associated receptor and is accompanied by minor forms with M_r values ranging from 104 000 to 180 000. The presence of receptor fragments in biological fluids indicates that shedding is one of the mechanisms contributing to the turnover of the M6P/IGF II receptor and that receptor fragments are part of the heterogenous group of serum proteins which bind IGF II.

INTRODUCTION

The cation-independent mannose 6-phosphate (M6P) receptor mediates endocytosis of extracellular lysosomal enzymes and transfer of endogenous lysosomal enzymes to lysosomes (for review see von Figura & Hasilik, 1985). Several observations suggest that the turnover of this receptor, which exposes more than 90 % of its mass at the external side of the membrane (Sahagian & Steer, 1985; von Figura *et al.*, 1985; Morgan *et al.*, 1987), occurs via non-lysosomal mechanisms and may involve shedding of receptor fragments (Creek & Sly, 1983; Sahagian, 1984). Receptor fragments may affect the extracellular lifetime of secreted lysosomal enzymes, if the fragments retain their binding activity. Therefore, we examined biological fluids for the presence of receptor fragments which retain their M6P-binding activity. While this study was in progress, it became apparent that the cation-independent M6P receptor also binds insulin-like growth factor II (IGF II) and is identical with the IGF II receptor (Morgan *et al.*, 1987).

In the present communication we report the presence of immunoreactive fragments of the M6P/IGF II receptor in human serum and human urine which bind M6P and IGF II.

MATERIALS AND METHODS

Phosphomannan from *Hansenula holstii* was a gift from Dr M. Slodki (U.S.D.A. Northern Regional Research Center, Peoria, IL, U.S.A.). AH-Sepharose 4B was from Pharmacia. M6P (barium salt) and glucose 6-phosphate (sodium salt) were obtained from Sigma Chemical Co. Bovine serum albumin, Protein A and *p*-nitrophenyl phosphate were purchased from Serva. Disuccinimidyl suberate (DSS) was from Pierce Chemical Co. Auro dye forte was from Janssen Life Science.

Standard proteins for molecular mass determination were purchased from Bio-Rad. Goat anti-(rabbit IgG)-alkaline phosphatase conjugate was from Sigma and TAGO (Burlingame, CA, U.S.A.). Homogeneous 215 kDa M6P/IGF II receptor from human liver and antisera in rabbit or in goat were prepared as described earlier (Geuze *et al.*, 1984). IgGs were isolated by using Protein A-Sepharose 4B (Pharmacia) and affinity-purified antibodies using receptor-Sepharose 4B (Geuze *et al.*, 1984). IGF II (preparation II/4 and 1932) was isolated from human serum (Rinderknecht & Humbel, 1976). IGF II was iodinated with Na^{125}I using the chloramine-T procedure (Zapf *et al.*, 1981). The specific activity of ^{125}I -IGF II was 150 000 c.p.m./ng. Other reagents used in this study were of analytical grade. Protein was determined (Peterson, 1977) using bovine serum albumin as a standard.

Purification of the M6P-receptor fragments from human serum and urine

M6P-binding proteins from either source were purified with phosphomannan core-Sepharose 4B affinity matrix (Stein *et al.*, 1987). In a typical purification procedure 25 ml of human serum or proteins precipitated from 5 litres of human urine with 70 % saturated $(\text{NH}_4)_2\text{SO}_4$ were extensively dialysed against imidazole buffer (50 mM-imidazole, pH 7.0/150 mM-NaCl/10 mM- MnCl_2 /5 mM-sodium β -glycerophosphate/0.05 % Triton X-100). The clear supernatant was passed through the affinity column (15 ml bed vol.). The column was extensively (2–4 litres) washed with the above buffer. The non-specifically bound proteins were eluted with 20 ml of imidazole buffer containing 2 mM-glucose 6-phosphate. Specifically bound proteins were eluted with 20 ml of imidazole buffer containing 2 mM-M6P. The proteins re-

Abbreviations used: M6P, mannose 6-phosphate; DSS, disuccinimidyl suberate; IGF II, insulin-like growth factor II; ELISA, enzyme-linked immunosorbent assay.

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covered were concentrated about 30-fold on an Amicon ultrafiltration assembly using a PM-10 membrane.

Electrophoresis and Western blotting

SDS/polyacrylamide-gel electrophoresis in 7.5% acrylamide gel under non-reducing conditions was performed according to Laemmli (1970). The polypeptides were transferred to nitrocellulose at 10 V/cm for 6 h and characterized by staining with Auro dye forte or with affinity-purified antibodies against the M6P/IGF II receptor (Conary *et al.*, 1987).

ELISA

ELISA was performed as described (Frackelton *et al.*, 1976) with some modification. Wells in microtitre plates were coated for 4 h at 37 °C with 50 µl of goat anti-receptor serum or goat preimmune serum, which were diluted (1:1600) with 25 mM-Tris/HCl (pH 7.0). The wells were saturated with buffer A [5% (w/v) fat-free milk powder in 50 mM-Tris/HCl (pH 7.8) containing 2 mM-CaCl₂/0.05% Triton X-100/0.02% sodium azide] overnight. Further coating with serial dilutions of purified M6P/IGF II receptor (1.25–20 ng/50 µl) or human serum (diluted in buffer A) was carried out at 37 °C for 2 h. The wells were washed with buffer B (50 mM-Tris/HCl, pH 7.4/0.5 M-NaCl/0.1% Triton X-100) and saturation with buffer A was repeated for 30 min at 37 °C. Subsequently, affinity-purified rabbit anti-receptor antibodies (1 µg of IgG/ml in buffer A) were added for 2 h at 37 °C. Following the washing with buffer B and saturation with buffer A, the plates were incubated for 2 h with goat anti-(rabbit IgG)-alkaline phosphatase conjugate. The wells were washed with buffer B followed by buffer C (0.1 M-Tris/HCl, pH 9.5/0.1 M-NaCl/2 mM-MgCl₂). The plate was finally incubated with *p*-nitrophenyl phosphate (1 mg/ml in buffer C) for 30 min at room temperature and absorbance at 405 nm was recorded.

IGF II binding assay

The binding of receptor with ¹²⁵I-IGF II was carried out in microtitre plates with some modification of the procedure previously described (Morgan & Roth, 1985). Wells were coated with 1 µg of Protein A in 50 µl of 25 mM-Tris/HCl (pH 7.0) for 5 h at 37 °C and were washed five times with 200 µl of Hepes buffer (50 mM-Hepes, pH 7.6/150 mM-NaCl/0.1% Tween 20/0.1% Triton X-100/0.1% bovine serum albumin). All following incubations and washings were done with this Hepes buffer. Anti-(receptor IgG), 1.25 µg in 25 µl, was added to each well. After incubation for 2 h at 37 °C the wells were washed five times, followed by an incubation with purified receptor or receptor fragments for 3 h at 37 °C. After washing five times, ¹²⁵I-IGF II (20 000 c.p.m./well) was added. After incubation for 3 h at room temperature the wells were washed five times, dried, cut, and counted. A control without receptor was performed in each experiment in order to subtract the non-specific binding.

Crosslinking of IGF II with receptor

Acetone precipitate of 1 µg of purified receptor or 0.2–0.3 µg of human serum or urine receptor fragments (quantification based on immunoreactivity) was solubilized in 37 µl of 50 mM-sodium phosphate, pH 7.4, containing 150 mM-NaCl/0.1% Triton X-100/0.1% bovine serum albumin and incubated for 20 min at 4 °C

with or without 10⁻⁶ M-IGF II. Then ¹²⁵I-IGF II, 13 µl (250 000 c.p.m.), was added and incubated for 3 h at 4 °C. Crosslinking of bound IGF II with receptor was performed with 0.6 mM-DSS in the reaction mixture by incubating for 15 min at 4 °C. The reaction was stopped with 5 µl of 1 M-Tris/HCl, pH 7.4, 2.75 µl of 20% SDS and 3 µl of glycerol (100%). The reaction mixture was heated at 95 °C for 5 min and the samples were subjected to SDS/polyacrylamide-gel electrophoresis followed by autoradiography. Alternatively the reaction was stopped with 5 µl of 1 M-Tris/HCl, pH 7.4, and the samples were immunoprecipitated as described (von Figura *et al.*, 1985).

RESULTS

Immunoreactive fragments of the M6P/IGF II receptor that bind M6P

Human serum was passed over a phosphomannan core-Sephrose 4B column. The material eluted from the affinity matrix with glucose 6-phosphate (irrelevant sugar phosphate) and with M6P was concentrated,

Fig. 1. Receptor-related polypeptides in human serum and urine

Portions of the glucose 6-phosphate (lane 1 and 3) and the M6P (lanes 2, 4 and 5–8) eluates corresponding to 1.25 ml of serum or 250 ml of urine, respectively, and 0.1 µg of purified liver M6P/IGF II receptor (lane 9) were separated by SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose and stained for protein with Auro dye forte (lanes 1 and 2) or analysed for polypeptides crossreacting with affinity-purified antibodies against the M6P/IGF II receptor (lanes 3–9). Lanes 1–4 represent samples from one purification, lanes 6–8 are the M6P eluates of three independent purifications (serum purifications I–III). The relative molecular masses of the immunoreactive polypeptides are indicated on the right.

Fig. 2. Crosslinking of IGF II with receptor fragments

The glucose 6-phosphate (lanes 1, 3 and 5) and the M6P (lanes 2, 4, 6, 9 and 10) eluates of two serum purifications (I, II) and one urine purification as well as purified M6P/IGF II receptor (lanes 7, 8) were incubated with ^{125}I -IGF II and subjected to crosslinking in the presence (lanes 1–8 and 10) or absence (lane 9) of DSS. Sample in lane 8 contained an excess of unlabelled IGF II (10^{-6} M). After separation by SDS/polyacrylamide-gel electrophoresis, lanes 1–8 were subjected to autoradiography, and lanes 9 and 10 to Western blotting (see Fig. 1). The M_r 205000 and M_r 185000 receptor fragments, which bind IGF II, are marked. The IGF II-binding material that migrates more slowly than the M_r 205000 receptor fragment consists of aggregated receptors formed during crosslinking (see lanes 9 and 10). Other labelled material (marked by x) represents polypeptides that bind IGF II unspecifically (not displaceable by unlabelled IGF II, see lane 8) and are unrelated to the receptor. These polypeptides derive from the albumin preparation (not shown). Free ^{125}I -IGF II is marked by xx.

separated by SDS/polyacrylamide-gel electrophoresis, and transferred to nitrocellulose. The protein pattern in both fractions was comparable (Fig. 1, lanes 1 and 2). In the M6P eluate several polypeptides (M_r 104000–205000) were detectable, which reacted with an affinity-purified antibody against the M6P/IGF II receptor and were absent from the glucose 6-phosphate eluate (Fig. 1, lanes 3 and 4). Based on their size, binding and immunological properties, these polypeptides are considered to be fragments of the M6P/IGF II receptor. In different preparations the relative amount of the receptor fragments was subject to some variations (see Fig. 1, lanes 6–8). The M_r 205000 polypeptide was always the predominant receptor fragment. The purified receptor from human liver has an apparent molecular mass of 215000 (Fig. 1, lane 9). In human urine the receptor fragments are more homogenous and consist of a major M_r 208000 and a minor M_r 118000 species (Fig. 1, lane 5).

The amount of immunoreactive polypeptides (irrespective of their binding properties) in serum was determined with an ELISA that allowed the detection of ≥ 50 ng of

Fig. 3. Crosslinking of IGF II with receptor fragments and immunoprecipitation

The M6P eluate (serum purification I) was incubated with ^{125}I -IGF II in the absence (lanes 1, 3 and 4) or presence of an excess (10^{-6} M) of IGF II (lane 2) and crosslinked with DSS. Total incubation mixtures (lanes 1 and 2), immunoprecipitates (lanes 3 and 4) and supernatants from immunoprecipitates (lanes 5 and 6) were subjected to SDS/polyacrylamide-gel electrophoresis. Precipitation was performed either with anti-(M6P/IGF II receptor Ig) (lanes 3 and 5) or control rabbit Ig (lanes 4 and 6). The M_r 205000 and M_r 185000 receptor fragments are indicated. (For material labelled with x or xx see Fig. 2.)

receptor polypeptide/ml of serum. In ten individuals (age 13–70 years) the amount of immunoreactive fragments varied between 1.1 and $1.7 \mu\text{g}$ of receptor/ml of serum (mean 1.4). The amount of immunoreactive fragments recovered after affinity chromatography accounted for approx. 10% of the immunoreactive polypeptides in serum, suggesting that only part of the circulating receptor-related polypeptides retain the M6P-binding site.

IGF II binding of receptor fragments

Receptor purified from liver membranes and receptor fragments isolated from serum and urine according to their ability to bind M6P were examined for their ability to bind IGF II. Purified receptor as well as the M6P and glucose 6-phosphate eluates from serum and urine were incubated with ^{125}I -IGF II, crosslinked and analysed by gel electrophoresis and autoradiography (Fig. 2). The purified receptor bound ^{125}I -IGF II. The specificity of the binding was demonstrated by the displacement of ^{125}I -IGF II by an excess of IGF II (Fig. 2, lanes 7 and 8). The glucose 6-phosphate eluates of serum and urine did not contain IGF II-binding proteins. In the M6P eluates of serum and urine a major M_r 205000, and a less abundant M_r 185000, IGF II-binding polypeptides were found (Fig. 2, lanes 2, 4 and 6). It should be noted that crosslinking (independent of IGF II binding) led to aggregation of receptor fragments (Fig. 2, lanes 9 and 10). The binding of ^{125}I -IGF II to the M_r 205000 and 185000 polypeptides was inhibited by an excess of IGF II (Fig. 3, lanes 1 and 2). The relation of the two

Fig. 4. IGF II binding by immobilized receptor fragments

Purified M6P/IGF II receptor (○) or dilutions of the glucose 6-phosphate (■) and M6P (●) eluates of serum purification III were assayed for IGF II binding in a solid-phase assay (see the Materials and methods section).

IGF II-binding polypeptides to the M6P/IGF II receptor is shown by their precipitation with receptor antibodies (Fig. 3, lanes 3–6). In Figs. 2 and 3 several other polypeptides are seen, which bind ^{125}I -IGF II. Binding to these polypeptides was not inhibited by an excess of unlabelled IGF II and is therefore considered to be unspecific. Furthermore, these polypeptides are unrelated to the receptor (Fig. 3) and the same IGF II-binding polypeptides were seen in a control lacking the receptor (not shown). They are likely to be derived from the albumin containing buffer.

A solid-phase assay was used to quantify the receptor fragments by virtue of their IGF II-binding activity. Purified M6P/IGF II receptor was immobilized and incubated with ^{125}I -IGF II. The amount of bound IGF II was proportional to the amount of receptor over a range of 2.5–80 ng of receptor protein (Fig. 4). The glucose 6-phosphate and M6P eluates from human serum were assayed for IGF II binding with the solid-phase assay. IGF II binding was detectable only in the M6P eluate (Fig. 4). An equivalent of 1 ml of serum, which contained 98 ng of immunoreactive material, had the same binding capacity as 26 ng of purified liver receptor.

DISCUSSION

Human serum and human urine contain polypeptides that bind M6P, IGF II and crossreact with antibodies against the M6P/IGF II receptor. These polypeptides are considered to represent extracellular forms of the M6P/IGF II receptor. The major receptor forms in serum and urine are about 10 kDa smaller in size than the intact receptor. The cytosolic and transmembrane domain of the receptor account for about 10% of its total mass (Morgan *et al.*, 1987). The major circulating receptor fragment may therefore represent a truncated receptor that has lost its cytosolic and transmembrane domain, but retained most of its extracytosolic portion.

The source of the receptor fragments in serum and urine is not clear. It is conceivable that alternative processing of the receptor mRNA results in formation of truncated receptors, which are secreted due to the lack of a transmembrane domain. The sensitivity of the extra-cytosolic part of the receptor to proteases, however, renders it likely that the receptor fragments arise from proteolytic cleavage of membrane-associated receptors.

The receptor fragments retaining the M6P-binding site may represent only a subpopulation of the receptor fragments present in biological fluids. This was suggested by the observation that the amount of immunoreactive receptor fragments in serum exceeds the amount of receptor fragments recovered after affinity chromatography by about 10-fold. A similar consideration may apply for the receptor fragments retaining the IGF II-binding site.

The major M_r 205 000 receptor fragment, which was purified on the M6P affinity matrix, had the same size as the major IGF II-binding receptor fragment. This suggests that the M_r 205 000 receptor fragment contains binding sites for both ligands. However, the binding of IGF II and M6P occurs at different sites, since the two ligands do not compete for binding (T. Braulke, unpublished work). It is therefore conceivable that receptor fragments exist which retain the binding activity for only one type of ligand.

It is obvious from the present results that M6P/IGF II receptor fragments belong to the heterogeneous group of IGF II-binding proteins in serum (for review see Rechler & Nissley, 1985). Some of these binding proteins are under the control of growth hormone (Moses *et al.*, 1976). The molecular masses of the binding proteins characterized so far were significantly lower than those of the major receptor fragments (Rechler & Nissley, 1985). During completion of this manuscript Kiess *et al.* (1987) reported an M_r approx. 210 000 IGF II-binding protein in rat serum, which crossreacts with antibodies against the IGF II receptor and is approx. 10 kDa smaller than the soluble IGF II receptor from rat placenta membranes. We believe that the IGF II receptor in rat serum corresponds to the major M_r 205 000 M6P/IGF II receptor fragment in human serum and urine.

It has been observed that the half-life of the M6P/IGF II receptor, which is about 1 day in various cultured cells, is not affected by inhibition of lysosomal proteolysis (Creek & Sly, 1983). This suggested a non-lysosomal pathway for receptor turnover. Chinese hamster ovary cells released immunoreactive receptor fragments if the medium was enriched in lysosomal enzymes (Sahagian, 1984). The present results suggest that the release of soluble receptor fragments may be a major mechanism determining the turnover of membrane-associated M6P/IGF II receptors. Receptor fragments that retain their M6P-binding site might bind extracellular lysosomal enzymes. This would prevent the M6P-dependent reuptake of the secreted lysosomal enzymes and prolong their extracellular lifetime. A mechanism allowing proteolytic cleavage of cell-associated receptors concomitant with the secretion of lysosomal enzymes would be particularly suitable for conditions such as tissue repair, inflammation or tumour invasion, where high extracellular concentrations of lysosomal enzymes are found. Limited proteolysis would reduce the probability of reuptake of extracellular lysosomal enzymes in a dual fashion by decreasing the number of endocytotic recep-

tors and by masking the M6P-recognition marker on the secreted lysosomal enzymes.

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