Mannose 6-phosphate-specific receptor is a transmembrane protein with a C-terminal extension oriented towards the cytosol

Kurt VON FIGURA, Volkmar GIESELMANN and Andrej HASILIK
*Physiologisch-Chemisches Institut der Westfälischen Wilhelms-Universität, Waldeyerstrasse 15,
D-4400 Münster, Federal Republic of Germany*

(Received 25 October 1984/Accepted 13 November 1984)

The portion of the mannose 6-phosphate receptor (nominal M_r 180000 under non-reducing conditions) protruding at the external side of the plasma membrane of fibroblasts and HepG2 cells is susceptible to trypsin. A series of membrane-bound fragments smaller in M_r by 20000–65000 is obtained after incubation of cells with trypsin. When membranes from fibroblasts and HepG2 cells are incubated with trypsin or *Staphylococcus aureus* proteinase, the receptor is degraded to a single membrane-bound product smaller in M_r by about 9000. In the presence of 0.1% Triton X-100 extensive degradation of the receptor by trypsin is observed. Furthermore, the receptor in isolated membranes is sensitive to carboxypeptidase Y, which causes a decrease in M_r by about 5000 and 9000 in the absence or presence of detergent, respectively. Mannose 6-phosphate receptor appears to be a transmembrane protein with multiple trypsin-sensitive sites within its larger external (luminal) and smaller C-terminal (cytosolic) portions of the molecule.

In fibroblasts and several other cell types MPR is essential for transport of lysosomal enzymes from the Golgi complex and the cell surface to lysosomes (Sly & Fischer, 1982; Hasilik *et al.*, 1983). The MPR binds phosphorylated high-mannose oligosaccharides in lysosomal enzymes. The lysosomal enzyme-MPR complexes formed in the Golgi organelle and at the plasma membrane are translocated into prelysosomal organelles from where the lysosomal enzymes are transported into lysosomes, and the MPR is returned to the Golgi complex and the plasma membrane (Sahagian & Neufeld, 1983; Geuze *et al.*, 1984a,b; von Figura *et al.*, 1984).

Thus, targeting of ligand-occupied and free MPR involves several sorting steps and is likely to depend on the interaction of MPR with other cellular structures. The MPR is synthesized as a glycosylated membrane protein. Under reducing conditions the apparent M_r is 215000 and under non-reducing conditions 180000 (Sahagian & Neufeld, 1983; Creek & Sly, 1983; Goldberg *et al.*, 1983; von Figura *et al.*, 1984). From the function of the receptor it may be inferred that the lysosomal

enzyme binding site of the MPR is exposed at the luminal side in the Golgi complex and the external side at the plasma membrane. In this study evidence is provided that MPR is a transmembrane protein with a C-terminal extension at the cytosolic side of membranes.

Materials and methods

Cell culture

Human diploid skin fibroblasts and the human hepatoma HepG2 cell line were maintained at 37°C under air/CO₂ (19:1) in Eagle's minimal essential medium, supplemented with antibiotics, non-essential amino acids, and 20% (v/v) fetal calf serum (Boehringer Mannheim) as described (Cantz *et al.*, 1972). The HepG2 cell line was kindly provided by Dr. A. Schwartz, Department of Pediatrics, Harvard University, Boston, MA, U.S.A.

Labelling of cells

Fibroblasts grown to confluency in 25 cm² or 75 cm² flasks were labelled with 30–150 μ Ci of L-[³⁵S]methionine (sp. radioactivity 1.2 Ci/nmol) for 16 h as described (von Figura *et al.*, 1983).

Abbreviations used: MPR, mannose 6-phosphate receptor; SDS, sodium dodecyl sulphate.

Incubation of labelled cells with trypsin

After labelling, cells were washed twice with Hank's balanced salt solution and incubated with 1 ml of 0.1% trypsin (Boehringer Mannheim) in serum-free medium (Gorham & Waymouth, 1965; as formulated in the Gibco catalogue) containing 20 mM-Hepes, pH 7.4, and 5 mM-mannose 6-phosphate. After incubation for 0–30 min at 37°C, 1.5 ml of ice-cold 0.15 M-NaCl containing 0.1% egg white trypsin inhibitor and 0.5% bovine serum albumin were added and cells were transferred into tubes. Adherent cells were collected by scraping. The cell-free supernatant containing the trypsin-released material, and the cells, were obtained by centrifugation. The cells were ruptured by ultrasonication in 0.1 M-sodium acetate, pH 6.0, containing 0.2 M-NaCl, 1 mM-phenylmethanesulphonyl fluoride, 5 mM-iodoacetamide and 1 mM-EDTA. After centrifugation for 20 min at 50 000g, a soluble and a membrane fraction were obtained. The trypsin-released material and the soluble fraction of cells were mixed with an equal volume of buffer A (10 mM-sodium phosphate, pH 7.4, containing 0.15 M-NaCl, 2% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 4% bovine serum albumin, 2 mM-phenylmethanesulphonyl fluoride, 10 mM-iodoacetamide and 2 mM-EDTA). The pellet was solubilized by ultrasonication in 0.8 ml of buffer B (buffer A diluted 1:1 with water).

Digestion of membranes

Cells labelled for 16 h with 0.15 mCi of [³⁵S]methionine in a 75 cm² flask were washed twice with ice-cold 0.9% NaCl, collected by scraping and suspended in 3 ml of 0.1 M-sodium acetate, pH 6.0, containing 0.2 M-NaCl. After ultrasonication (three bursts of 5 M each) and centrifugation for 20 min at 50 000g the pellet was suspended in 20 mM-sodium phosphate, pH 7.4, containing 0.15 M-NaCl by 20 strokes in a tight-fitting Dounce homogenizer. Aliquots (0.1 ml) were incubated in the absence or presence of 0.1% Triton X-100 with 0.05% trypsin or 0.025% V8 proteinase (Miles) or 0.025% carboxypeptidase Y (Hasilik & Tanner, 1976) in a final volume of 0.12 ml. Incubation with carboxypeptidase was performed also at pH 5.4 (adjusted with 1 M-sodium acetate, pH 4.0). After incubation for 45 min at 37°C, reaction was terminated by chilling on ice and adding 4 µl of 0.25 M-phenylmethanesulphonyl fluoride, 60 µl of 0.5% egg white trypsin inhibitor in 0.15 M-NaCl and 185 µl of buffer A. After ultrasonication the mannose 6-phosphate receptor was isolated by immunoprecipitation.

Immunoprecipitation

The samples were mixed with 10 mg of Immuno-

precipitin (formalin-fixed *Staphylococcus aureus* cells; Bethesda Research Laboratories) and kept for 10 min on ice. The Immunoprecipitin was removed by centrifugation. Preadsorption of supernatants with Immunoprecipitin was repeated twice. The final supernatant was cleared by centrifugation for 1 h at 35 000g and mixed with 8 µg of affinity-purified rabbit antibody against human liver MPR (von Figura *et al.*, 1984). After incubation overnight at 4°C, 15 µl of 10% Immunoprecipitin was added. The Immunoprecipitin was pretreated by heating for 30 min at 85°C in buffer C [50 mM-Tris/HCl, (pH 7.4)/0.15 M-NaCl/5 mM-EDTA], followed by heating for 5 min at 95°C in buffer C containing 1% SDS. The Immunoprecipitin was washed three times with buffer C containing 0.5% bovine serum albumin and 0.5% Triton X-100, twice with phosphate-buffered saline and stored in phosphate-buffered saline containing 1% bovine serum albumin and 0.04% NaN₃. After 30 min on ice the Immunoprecipitin was collected and suspended in 200 µl of buffer B containing 5 mM-mannose 6-phosphate. After incubation for 10 min on ice, the Immunoprecipitin was collected by centrifugation, washed twice with 0.8 ml of buffer B and then with 0.8 ml of 1 mM-sodium phosphate, pH 7.4, containing 0.015 M-NaCl, and finally heated for 5 min at 95°C in 0.125 M-Tris/HCl, pH 6.8, containing 1% SDS, 10% glycerol and, where indicated, 10 mM-dithiothreitol. The solubilized material was separated by electrophoresis in 12.5%, 8% or 6% polyacrylamide gels (Laemmli, 1970) and the radioactivity was visualized by fluorography (Laskey & Mills, 1975). Radioactive protein standards were from New England Nuclear.

Results

Susceptibility of cell-surface MPR to trypsin

Fibroblasts were labelled for 16 h with [³⁵S]methionine and then incubated for up to 30 min at 37°C with 0.1% trypsin. A cell-free supernatant containing the trypsin-released material and the cell pellet were obtained by centrifugation. The cells were broken by ultrasonication and separated into a soluble and a membrane fractions. The trypsin-released material and the soluble and membrane fractions of the cells were analysed for MPR polypeptides. Polypeptides cross-reacting with a polyclonal antiserum against MPR were detectable only in the membrane fractions of trypsinized and untrypsinized cells. Between 60 and 70% of the MPR was degraded by trypsin. Among the membrane-bound fragments, those with apparent *M_r* values of 160 000, 140 000, 130 000, 118 000 and 105 000 (Fig. 1) were prominent. In addition, minor fragments with *M_r* values ranging from

Fig. 1 *MPR in fibroblasts treated with trypsin*
MPR was isolated from the membrane fraction of fibroblasts that had been incubated for 0, 10, 20, and 30 min at 37°C with 0.1% trypsin. The immunoprecipitates were solubilized and separated in a 12.5% polyacrylamide gel under non-reducing conditions. The position of MPR (→) and trypsin fragments of MPR (—) is indicated.

15000 to 45000 were found. Comparable products were obtained when human hepatoma HepG2 cells were subject to trypsin treatment (results not shown).

Susceptibility of MPR in isolated membranes to trypsin, V8 proteinase and carboxypeptidase Y

A membrane fraction was prepared from fibroblasts and HepG2 cells labelled for 16h with [³⁵S]methionine. The membranes were incubated for 30 min at 37°C with trypsin, V8 proteinase and carboxypeptidase Y in the absence of 0.1% Triton X-100 and MPR was isolated from the whole digest

Fig. 2. *Treatment of membranes from fibroblasts with proteinases*

Membranes were incubated at pH 7.4 for 45 min at 37°C with trypsin (Try), *Staphylococcus* V8 proteinase (V8), or carboxypeptidase Y (CY) in the absence or presence of 0.1% Triton X-100. Membranes in lanes 8 and 9 were incubated at pH 5.4. The immunoprecipitates were solubilized and separated in a 8% polyacrylamide gel under non-reducing conditions. The position of MPR (→) is indicated.

(Fig. 2). Trypsin and V8 proteinase caused a decrease in the apparent M_r by about 9000. A decrease in size by about 5000 was achieved by treatment with carboxypeptidase Y at pH 5.4. At pH 7.4, which is less favourable for the action of carboxypeptidase Y (Hayashi, 1976), a decrease in size was hardly detectable. In the presence of Triton X-100 MPR was degraded by trypsin to several smaller fragments (apparent M_r values ranging from 60000 to 140000). Most of the MPR-associated radioactivity, however, was lost, indicating that degradation was accompanied by a loss of immunoreactivity. Upon addition of Triton X-100, the decrease in M_r caused by digestion with carboxypeptidase Y was about 9000 compared with 5000 in the absence of detergent (Fig. 2). Triton X-100 had no effect on the degradation of MPR by V8 proteinase (Figs. 2 and 3). The membrane-bound fragments of MPR obtained after digestion of membranes from HepG2 cells with trypsin, V8 proteinase and carboxypeptidase Y had the same apparent M_r values as those observed in fibroblasts (Fig. 3).

Fig. 3. Treatment of membranes from HepG2 cells with proteinases

MPR was immunoprecipitated from membranes incubated at pH 7.4 with or without proteinases in the absence or presence of Triton X-100. Membranes in lane 11 were incubated at pH 5.4. The immunoprecipitates were solubilized and separated in a 8% (lanes 1-7) or 6% (lanes 8-11) polyacrylamide gel under non-reducing conditions. The position of MPR (→) is indicated.

Discussion

A minor fraction of MPR is located at the cell surface. In CHO cells about 10% of the total MPR fractionates with plasma membrane markers (Sahagian & Neufeld, 1983). The observed degradation of up to 70% of the MPR by exposure of fibroblasts to trypsin depends, therefore, on translocation of MPR from internal membranes to the cell surface during the incubation. If fibroblasts are incubated with trypsin at 4°C, all surface MPR and less than 10% of total MPR is degraded, indicating that translocation of MPR from internal sites to the cell surface is temperature-dependent (K. von Figura, V. Gieselmann & A. Hasilik, unpublished work). The apparent M_r of the major membrane-bound fragments obtained after trypsin treatment of cells varies from 105000 to 160000. The formation of distinct fragments indicates the existence of several trypsin-sensitive sites, which might be contained either within one large extension or within separate domains protruding at the external side of the plasma membrane. From the size of the fragments it may be inferred that the major part of MPR is exposed at the external side of the plasma membrane and, correspondingly, at the luminal side of intracellular membrane-delimited organelles.

Treatment of membranes with trypsin or V8 proteinase leads to an apparently homogenous product that is smaller in size by only 9 kDa. This product is considerably larger than any of those observed after treatment of intact cells. In the presence of detergent the MPR in membranes is rapidly degraded by trypsin to multiple products, which lack cross-reactivity with the antibody. As addition of detergent in the presence of V8 proteinase has no effect on the degradation of MPR, we assume only one or several adjacent proteinase-sensitive sites. The relative resistance of MPR in the membrane preparation to proteolysis in the absence of the detergent indicates that this preparation consists largely of closed vesicles with the extracytosolic (external and luminal) surface oriented to the interior. The similarity in the reduction in size by about 9 kDa after digestion with trypsin and V8 proteinase indicates that only a small part of the receptor is exposed at the cytosolic side of the membranes. This cytosolic extension forms the C-terminus of the MPR. This was shown by the susceptibility of MPR in isolated membranes to carboxypeptidase Y, which caused a reduction in apparent M_r of the MPR by about 5000.

The present results show that the MPR in fibroblasts and HepG2 cells is a transmembrane protein. The extension protruding at the external (luminal) side contains the binding site(s) for mannose 6-phosphate residues. The small cytosolic moiety, representing the C-terminus of the MPR, may play an important role in targeting of the MPR. Transport of lysosomal enzymes depends on transfer of MPR-lysosomal enzyme complexes from either the Golgi apparatus or the cell surface to prelysosomal organelle(s) and on return of free receptors to the Golgi complex or plasma membrane (Sahagian & Neufeld, 1983; Geuze *et al.*, 1984b; von Figura *et al.*, 1984). It should be of interest to examine the role of the cytosolic moiety of MPR in the targeting of lysosomal enzymes.

The help of Mrs H. Flentje is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

References

- Cantz, M., Dresse, H., Barton, R. W. & Neufeld, E. F. (1972) *Methods Enzymol.* **28**, 883-897
- Creek, K. E. & Sly, W. S. (1983) *Biochem. J.* **214**, 353-360
- Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Hasilik, A. & von Figura, K. (1984a) *J. Cell Biol.* **98**, 2047-2054
- Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Peppard, J., von Figura, K., Hasilik, A. & Schwartz, A. L. (1984b) *Cell* **37**, 195-204
- Goldberg, D. E., Gabel, C. A. & Kornfeld, S. (1983) *J. Cell Biol.* **97**, 1700-1706

- Gorham, L. W. & Waymouth, G. (1965) *Proc. Soc. Exp. Biol. Med.* **119**, 287–290
- Hasilik, A. & Tanner, W. (1976) *Antimicrob. Agents Chemother.* **10**, 402–410
- Hasilik, A., Pohlmann, R., Gieselmann, V., von Figura, K., Olsen, R. & Waheed, A. (1983) in *Protein Synthesis* (Abraham, A. K., Eickhom, T. S. & Pryme, J. F., eds.), pp. 349–370, Humana Press, Clifton, NY
- Hayashi, R. (1976) *Methods Enzymol.* **55**, 568–587
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- Sahagian, G. G. & Neufeld, E. F. (1983) *J. Biol. Chem.* **258**, 7121–7128
- Sly, W. S. & Fischer, H. D. (1982) *J. Cell Biochem.* **18**, 67–85
- von Figura, K., Steckel, F. & Hasilik, A. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6066–6070
- von Figura, K., Gieselmann, V. & Hasilik, A. (1984) *EMBO J.* **3**, 1281–1286