Biosynthesis and turnover of the phosphomannosyl receptor in human fibroblasts

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The phosphomannosyl receptor mediates intracellular targeting of newly synthesized acid hydrolases to lysosomes, and is also expressed as a pinocytosis receptor on the cell surface of fibroblasts. We have purified the phosphomannosyl receptor from bovine liver and produced rabbit antibodies to the bovine receptor. The antibodies partially blocked pinocytosis of human spleen β -glucuronidase by fibroblasts, a process mediated by the phosphomannosyl receptor. Affinity-purified antibodies to the phosphomannosyl receptor were used to study the biosynthesis and turnover of the receptor in human fibroblasts. Phosphomannosyl receptor immunoprecipitated after a 15 min pulse-labelling of fibroblasts with [35 S]methionine exhibited an identical mobility on sodium dodecyl sulphate/polyacrylamide gels as purified bovine liver phosphomannosyl receptor. Pulse-chase experiments for up to 3 days provided no evidence for changes in molecular weight attributable to post-translational processing of the phosphomannosyl receptor. Turnover studies determined that the half-life of the phosphomannosyl receptor in normal human fibroblasts was 24–29 h. The half-life of the receptor was slightly longer (32 h) in I-cell disease fibroblasts and normal fibroblasts exposed to leupeptin (32 h), slightly shorter in fibroblasts exposed to NH_4Cl (23 h) and saturating amounts of ligand (21 h) and unaffected in cells exposed to mannose 6-phosphate (24 h). These studies show that the turnover of the phosphomannosyl receptor in fibroblasts is very slow, in contrast with its rate of internalization in endocytosis, and that its rate of degradation is not greatly altered by a variety of agents that affect lysosomal protein turnover and/or receptor-mediated endocytosis. These results suggest that the degradative activities of the lysosomes do not play an important role in phosphomannosyl receptor turnover in cultured fibroblasts.

Pinocytosis and intracellular transport of acid hydrolases is mediated by a membrane-associated receptor that recognizes phosphomannosyl moieties on their high-mannose-type oligosaccharide chains (Neufeld & Ashwell, 1980; Sly & Fischer, 1982). Originally recognized as a pinocytosis receptor on the cell surface of fibroblasts (Kaplan *et al.*, 1977; Sando & Neufeld, 1977), phosphomannosyl receptors have since been reported in Swarm-rat chondro-

sarcoma cells (Rome & Miller, 1980), rat hepatocytes (Ullrich *et al.*, 1979; Sahagian *et al.*, 1981), bovine liver (Sahagian *et al.*, 1981; Fischer *et al.*, 1982), Chinese-hamster ovary cells (Robbins, 1979; Sahagian *et al.*, 1981), intracellular membranes isolated from fibroblasts (Fischer *et al.*, 1980a), all major tissues of the rat (Fischer *et al.*, 1980b) and several subcellular fractions isolated from rat liver (Fischer *et al.*, 1980b). The phosphomannosyl receptor is an integral membrane glycoprotein with an apparent molecular weight of 215 000 (Sahagian *et al.*, 1981; Steiner & Rome, 1982; Fischer *et al.*, 1982). It has been purified from bovine liver (Sahagian *et al.*, 1982) and Swarm-rat chondrosarcoma membranes (Steiner & Rome, 1982) by affinity chromatography on phosphomannan-

Abbreviation used: SDS, sodium dodecyl sulphate.

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Sephacrose. Recently we reported the purification of the phosphomannosyl receptor from bovine liver on an affinity column composed of glycoproteins secreted by the slime mold *Dictyostelium discoideum* (Fischer *et al.*, 1982). We have subsequently raised antibodies to the purified bovine liver phosphomannosyl receptor by immunization of rabbits.

In the present paper we have used affinity-purified antibodies to the phosphomannosyl receptor to study the biosynthesis and turnover of this receptor in human fibroblasts. Furthermore we have investigated the effects of agents known to alter the rate of degradation of proteins in lysosomes, or to alter the rate of pinocytosis and the route of intracellular traffic of lysosomal enzymes on the turnover of the phosphomannosyl receptor.

Materials and methods

Materials

Freund's adjuvants (complete and incomplete) were obtained from Difco Laboratories. L-[³⁵S]-Methionine (sp. radioactivity 953 Ci/mmol), carrier-free Na¹²⁵I (17 Ci/mg) and EN³HANCE were from New England Nuclear. NaB³H₄ (15 Ci/mmol) was from Research Products International. Nonidet P40 was from Bethesda Research Laboratories. DEAE Affi-Gel Blue and Immobead goat anti-(rabbit immunoglobulin) were from Bio-Rad Laboratories. Iodo-beads were from Pierce and X-Omat XAR-5 film was from Kodak. Protein A-bacterial adsorbent was from Miles Laboratories. NCS tissue solubilizer was from Amersham and Scintiverse liquid-scintillation mixture from Fisher. Aprotinin and leupeptin were from Sigma.

Cell cultures

Normal diploid human fibroblasts (IMR-91) and β -glucuronidase-deficient fibroblasts (GM-151) were obtained from the Human Mutant Cell Repository, Camden, NJ, U.S.A. I-cell disease fibroblasts from patient T. M. were obtained from the Division of Medical Genetics, University of Texas Health Science Center, Dallas, TX, U.S.A. All cultures were maintained at 37°C in CO₂/air (1:19) in minimal essential medium (Earle's medium; Gibco), supplemented with 15% heat-inactivated foetal-calf serum (Gibco), 1 mM-sodium pyruvate, 100 units of penicillin/ml and 100 μ g of streptomycin sulphate/ml.

Preparation of anti-(phosphomannosyl receptor) antibodies

Phosphomannosyl receptors were purified from bovine liver on an affinity column composed of glycoproteins secreted by *D. discoideum* as described

previously (Fischer *et al.*, 1982). The purified receptor (0.4 mg in 1.5 ml of 0.9% NaCl) was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into the thigh muscle of a New Zealand White rabbit. Before the first injection, pre-immune serum was obtained. At 2 weeks after the initial injection the rabbit was boosted with 0.4 mg of phosphomannosyl receptor (in 1.5 ml of 0.9% NaCl) that was mixed with an equal volume of Freund's incomplete adjuvant. At 2 weeks after the boost blood was collected from the ear vein.

Immune serum (5.0 ml) was dialysed at 4°C against two changes of 1 litre of buffer containing 0.02 M-Tris, 0.028 M-NaCl and 0.02% NaN₃, pH 7.3 ('Tris-NaCl buffer'). The dialysed serum was then applied at 4°C to a column of DEAE Affi-Gel Blue (35 ml) equilibrated with Tris-NaCl buffer. The column was eluted with Tris-NaCl buffer and 5.8 ml fractions were collected. Fractions 5–13, containing the first peak of protein, which constituted the major immunoglobulin G fraction, were pooled. An immunoglobulin G fraction was prepared from pre-immune serum in an identical manner.

An affinity-purified anti-(phosphomannosyl receptor) immunoglobulin G was prepared as follows. A column (0.7 cm $\times$ 10 cm) of phosphomannosyl receptors covalently linked to Affi-Gel 10, made as described previously (Fischer *et al.*, 1982), was equilibrated with Tris-NaCl buffer at 4°C. The anti-(phosphomannosyl receptor) immunoglobulin G fraction prepared as described above was applied to this column at a rate of 6.0 ml/h. The column was washed at 6.0 ml/h with Tris-NaCl buffer containing 0.5 M-NaCl until the A_{280} was 0.005. The column was then brought to room temperature and the specific antibodies to the phosphomannosyl receptor were eluted with 3 column volumes of 4.5 M-MgCl₂. The affinity-purified anti-(phosphomannosyl receptor) antibodies were dialysed extensively against Dulbecco's phosphate-buffered saline (PBS) containing 0.02% NaN₃ and concentrated by ultrafiltration on a PM 10 membrane filter (Amicon) to a final protein concentration of 230 μ g/ml. The affinity-purified anti-(phosphomannosyl receptor) antibodies could be stored at -70°C for at least 5 months without loss of activity.

Pulse-labelling of the phosphomannosyl receptor

Fibroblasts were grown to confluence in 35-mm Petri dishes (approx. 0.3–0.4 mg of protein/dish). Before radiolabelling, the cells were washed once with 2 ml of methionine-free Dulbecco's modified Eagle's (DME) medium containing 15% dialysed foetal-calf serum, 100 units of penicillin/ml and 100 μ g of streptomycin sulphate/ml. Methionine-free DME medium (2.0 ml) was then added and the

cells were starved for 1 h at 37°C in CO₂/air (1:19). The cells were then washed once with 2 ml of methionine-free DME medium and pulse-labelled for the times indicated in 1 ml of methionine-free DME medium containing 100 µCi of [³⁵S]methionine. After the pulse the cells were washed three times with 2.0 ml of regular DME medium supplemented with 5 mM-L-methionine and 10% foetal-calf serum and chased for the indicated times in 2.0 ml of this medium. After the indicated chase period, the cells were washed five times with 2.0 ml of PBS and lysed by the addition of 0.5 ml of 1% Nonidet P40 in PBS containing 10 µg of aprotinin/ml. The extract was scraped from the dish and left at 4°C for 20 min. The cell extract was clarified by centrifugation for 2 min in a Beckman Microfuge, the pellet discarded and the supernatant used immediately for immunoprecipitation of labelled phosphomannosyl receptor.

Immunoprecipitation of labelled phosphomannosyl receptor

The [³⁵S]methionine-labelled cell lysate (200 µl) was mixed with 20 µl of a solution containing 10% Nonidet P40, 10% sodium deoxycholate and 1% SDS in PBS and re-clarified by the addition of 100 µl of Immunobead goat anti-(rabbit immunoglobulin). After 1 h at room temperature the Immunobeads were pelleted by centrifugation for 1 min in a Beckman Microfuge. The cell extract was transferred to a new tube and antigen-antibody complexes were formed by the addition of 10 µl of affinity-purified anti-(phosphomannosyl receptor) antibody (2.3 µg of immunoglobulin G). After 2 h at room temperature, 300 µl of Immunobeads were added, and immune complexes were collected 2 h later by centrifugation for 1 min in a Beckman Microfuge. The Immunobeads were resuspended in 1.0 ml of 0.5% Nonidet P40/0.5% sodium deoxycholate/0.05% SDS in PBS, transferred to a new tube, washed three more times with 0.5 ml of the detergent-containing buffer and finally once with 0.5 ml of PBS. The immunoprecipitate was released from the Immunobeads by boiling for 5 min in 100 µl of sample buffer (75 mM-Tris, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 5% glycerol and 0.0015% Bromophenol Blue) containing 5 µg of purified bovine liver phosphomannosyl receptor (Fischer *et al.*, 1982).

SDS/polyacrylamide-gel electrophoresis of [³⁵S]-methionine-labelled immunoprecipitates

Polyacrylamide-gel electrophoresis was conducted under reducing conditions in 6% polyacrylamide gels containing 0.1% (w/v) SDS as described by Laemmli (1970). Electrophoresis was carried out at 40 mA per slab gel at room temperature for 2–2.5 h. Carrier unlabelled bovine liver phosphomannosyl receptor was visually detected by staining

with Coomassie Blue overnight. For fluorography, each slab gel was destained in 10% acetic acid for 3 h, soaked in EN³HANCE for 1 h, washed for 1 h with cold water, dried and exposed to Kodak XAR-5 film at –70°C. After radiographic exposure, the area of the dried slab gel containing the [³⁵S]methionine-labelled phosphomannosyl receptor (which co-migrated with the carrier bovine liver phosphomannosyl receptor) was carefully cut out, swollen in 50 µl of water and placed at 55°C for 2 h in 1.0 ml of NCS tissue solubilizer that was diluted 9:1 with water. Radioactivity was determined in 10 ml of Scintiverse liquid-scintillation mixture after neutralization with 50 µl of acetic acid.

Miscellaneous methods

Phosphomannosyl receptors purified from bovine liver were radiolabelled by reductive methylation with NaB³H₄ to a specific radioactivity of 16 000 c.p.m./µg as described by Tack *et al.* (1980). Bovine liver phosphomannosyl receptors were labelled to a specific radioactivity of 4.2×10^6 c.p.m./µg with ¹²⁵I using Iodo-beads according to the manufacturer's recommendations. Human spleen β-glucuronidase was purified as described previously (Natowicz *et al.*, 1979). Protein was measured as described by Lowry *et al.* (1951) with bovine serum albumin as standard. β-Glucuronidase was assayed fluorimetrically using 4-methylumbelliferyl β-D-glucuronide as the substrate (Fischer *et al.*, 1980a). One unit of activity is the amount of enzyme that catalyses the release of 1 nmol of 4-methylumbelliferone/h.

Results

Preparation and characterization of anti-(phosphomannosyl receptor) antibodies

Antibodies to the bovine phosphomannosyl receptor were raised by immunization of a rabbit with phosphomannosyl receptors purified from bovine liver. An immunoglobulin G fraction was isolated from the serum of the immunized rabbit by chromatography on DEAE Affi-Gel Blue. The specific antibodies to the phosphomannosyl receptor were subsequently purified from the immunoglobulin G fraction by affinity chromatography on a column of immobilized phosphomannosyl receptors. To assay the affinity-purified antibody for activity towards phosphomannosyl receptor we tested its ability to immunoprecipitate ¹²⁵I-labelled phosphomannosyl receptors isolated from bovine liver. As shown in Fig. 1, immune but not pre-immune immunoglobulin G precipitated the ¹²⁵I-labelled phosphomannosyl receptor. As would be expected, the addition of excess unlabelled bovine liver phosphomannosyl receptor inhibited the precipitation of the ¹²⁵I-labelled phosphomannosyl receptor in a

Fig. 1. Inhibition of immunoprecipitation of ^{125}I -labelled phosphomannosyl receptor by excess unlabelled phosphomannosyl receptor

^{125}I -Labelled phosphomannosyl receptor ($0.012\text{ }\mu\text{g}$) and the indicated concentrations of unlabelled phosphomannosyl receptor in $250\text{ }\mu\text{l}$ of 0.5% sodium deoxycholate/ 0.5% Triton X-100/ 0.05% SDS (final concentrations) in PBS were added to $2.3\text{ }\mu\text{g}$ of affinity-purified anti-(phosphomannosyl receptor) immunoglobulin G (●) or pre-immune immunoglobulin G (○). After 2 h at room temperature immune complexes were collected by the addition of $50\text{ }\mu\text{l}$ of a 10% (v/v) suspension of formalin-fixed *Staphylococcus aureus*. After 30 min the bacteria were pelleted in a Beckman Microfuge and washed three times with $300\text{ }\mu\text{l}$ of 0.5% sodium deoxycholate/ 0.5% Triton X-100/ 0.5% SDS (final concentrations) in PBS. The cells were then resuspended in 0.5 ml of the same buffer and radioactivity was determined by using a Packard auto-gamma scintillation spectrometer.

concentration-dependent manner as it competed with the labelled phosphomannosyl receptor for a limiting amount of anti-(phosphomannosyl receptor) antibody (Fig. 1). From this Figure we calculated that $2.3\text{ }\mu\text{g}$ of affinity-purified anti-(phosphomannosyl receptor) antibody was sufficient to quantitatively immunoprecipitate approx. $0.5\text{ }\mu\text{g}$ of phosphomannosyl receptor.

We next tested the activity of the anti-(phosphomannosyl receptor) antibodies for their ability to inhibit the pinocytosis of human spleen β -glucuronidase by human fibroblasts, an activity mediated by the phosphomannosyl receptor. As shown in Fig. 2, immunoglobulin G prepared from immune but not preimmune serum gave a concentration-dependent

Fig. 2. Inhibition of the uptake of β -glucuronidase by anti-(phosphomannosyl receptor) immunoglobulin G. Confluent 35-mm Petri dishes of β -glucuronidase-deficient fibroblasts were incubated for 1 h at 37°C in 1.0 ml of Eagle's minimal essential medium containing the indicated concentrations of pre-immune (○) or immune (●) immunoglobulin G. β -Glucuronidase (2000 units) was then added to each plate and after 2 h at 37°C the cells were chilled on ice and rinsed five times with 3 ml portions of PBS. Cell-associated β -glucuronidase was measured in cells disrupted with 0.5 ml of 1% sodium deoxycholate. The results are expressed as the percentages of cell-associated β -glucuronidase in experimental plates compared with control plates that did not receive any added immunoglobulin G.

inhibition of the uptake of β -glucuronidase by β -glucuronidase-deficient human fibroblasts. This result suggests that at least some of the anti-(phosphomannosyl receptor) antibodies bind the human receptor in such a way as to impair its function in enzyme pinocytosis. However, we have not determined if the binding of antibody to cell-surface phosphomannosyl receptors influences in any way the turnover of the receptor.

Biosynthesis and turnover of the phosphomannosyl receptor in human fibroblasts

We utilized the affinity-purified antibodies to the bovine phosphomannosyl receptor to study receptor biosynthesis in cultured human fibroblasts. The cells were pulse-labelled with $[^{35}\text{S}]$ methionine, chased for various times in the presence of excess unlabelled methionine, and the phosphomannosyl receptor immunoprecipitated from the cell lysate with the anti-(phosphomannosyl receptor) antibodies. The immunoprecipitate was analysed by SDS/polyacrylamide-gel electrophoresis followed by fluorography. As shown in Fig. 3(b), newly synthesized

Fig. 3. Pulse-chase labelling of phosphomannosyl receptor in normal human fibroblasts

Normal human fibroblasts were pulse-labelled for 15 min with [35 S]methionine and chased for zero (b), 30 (c), 60 (d) or 120 min (a and e) in the presence of excess unlabelled methionine. Detergent-solubilized cell extracts were immunoprecipitated with pre-immune immunoglobulin G (a) or affinity-purified anti-(phosphomannosyl receptor) immunoglobulin G (b–e) and the immunoprecipitates were subjected to SDS/polyacrylamide-gel electrophoresis and fluorography as described in the Materials and methods section. Lane (f) shows purified bovine liver phosphomannosyl receptor that was radiolabelled by reductive methylation (Tack *et al.*, 1980). The position of migration of the following molecular weight standards is indicated: myosin, 200 000 Da; β -galactosidase, 116 000 Da; phosphorylase b, 94 000 Da; bovine serum albumin, 68 000 Da; ovalbumin, 43 000 Da. The [35 S]methionine-labelled material migrating at the very bottom of the gel ran with the dye front and may represent low-molecular-weight proteins and peptides that non-specifically bind to the Immunobeads used in the immunoprecipitations (lanes a–e). The greater number of non-specific bands observed in the immunoprecipitate at the end of the 15 min pulse (lane b) when compared with chase periods of 30–120 min (lanes c–e) probably represent proteins that are very rapidly degraded and therefore do not contribute to the background at later chase periods.

human phosphomannosyl receptor immunoprecipitable after pulse-labelling normal human fibroblasts for only 15 min with [35 S]methionine exhibits an identical mobility on SDS/polyacrylamide-gel electrophoresis to that of radiolabelled bovine liver phosphomannosyl receptor (Fig. 3f), which migrates with an apparent molecular weight of 215 000 (Fischer *et al.*, 1982). Thus, the antibody prepared against the bovine receptor cross-reacts sufficiently with newly synthesized human receptor to allow studies of its biosynthesis and turnover. Further-

more, the apparent molecular weight of immunoprecipitable pulse-labelled receptor did not change detectably after chases of 30, 60 and 120 min (Figs. 3c, 3d and 3e, respectively). In fact, no apparent change in the molecular weight of pulse-labelled phosphomannosyl receptor was observed after chase periods of up to 3 days (results not shown). Apparent changes in molecular weight of approx. 10 000 Da would have been detectable. Importantly, the addition of pre-immune immunoglobulin G to [35 S]-methionine-labelled cell extracts did not precipitate a product with the same mobility of SDS/polyacrylamide-gel electrophoresis as the phosphomannosyl receptor (Fig. 3a).

Fig. 4. Turnover of [35 S]methionine-labelled phosphomannosyl receptor in normal and I-cell-disease fibroblasts

Normal (●) or I-cell (○) fibroblasts were pulse-labelled for 90 min with [35 S]methionine and chased for the indicated times in the presence of excess unlabelled methionine. Detergent-solubilized cell extracts were immunoprecipitated with affinity-purified anti-(phosphomannosyl receptor) antibodies and the immunoprecipitates were subjected to SDS/polyacrylamide-gel electrophoresis and fluorography as described in the Materials and methods section. Radioactivity that co-migrated with standard bovine liver phosphomannosyl receptor was quantified as described in the Materials and methods section. The data are presented as a semilogarithmic plot of the ratio of the radioactivity present in immunoprecipitable phosphomannosyl receptor at the end of the chase period (Receptor_t) to the radioactivity present in immunoprecipitable phosphomannosyl receptor at the end of the pulse (Receptor_0) versus the time of chase. Incorporation of [35 S]methionine into protein during the pulse-labelling period was 34.3×10^6 and 28.40×10^6 c.p.m. per mg of cell protein for normal and I-cell-disease fibroblasts respectively.

Fig. 5. Effect of agents that alter lysosomal enzyme activity and/or 'trafficking' on the turnover of the phosphomannosyl receptor

(a) Normal human fibroblasts were pulse-labelled for 90 min with [³⁵S]methionine and chased in the presence of excess unlabelled methionine only (●), or also in the presence of 10 mM-NH₄Cl (▲) or 20 μM-leupeptin (■). (b) Normal human fibroblasts were pulse-labelled for 90 min with [³⁵S]methionine and chased in the presence of excess unlabelled methionine only (●), or also in the presence of 20 000 units of β-glucuronidase/ml (▲) or 2 mM-mannose 6-phosphate (■). Radioactivity in phosphomannosyl receptor was determined by immunoprecipitation as described in the legend to Fig. 4 and in the Materials and methods section.

To study the turnover of the phosphomannosyl receptor in human fibroblasts we pulse-labelled the cells with [³⁵S]methionine for 90 min and then chased in the presence of excess unlabelled methionine for periods of up to 70 h. The immunoprecipitated phosphomannosyl receptor was analysed by SDS/polyacrylamide-gel electrophoresis followed by fluorography. Radioactivity co-migrating with carrier-unlabelled bovine phosphomannosyl receptor was quantified as described in the Materials and methods section. As shown in Fig. 4, the [³⁵S]-methionine-labelled phosphomannosyl receptor in normal human fibroblasts is relatively stable and disappears with a half-life of approx. 24 h. In I-cell disease fibroblasts, which are unable to phosphorylate their lysosomal enzymes and therefore contain no endogenous ligand for the receptor, the half-life of the phosphomannosyl receptor is 30 h or only slightly longer than the half-life determined for normal human fibroblasts (Fig. 4). Interpretation of the small differences in phosphomannosyl receptor turnover seen in Fig. 4 is not straightforward. Although the rates of incorporation of methionine into protein during the labelling period in the two cell lines were comparable (see the legend to Fig. 4), other factors such as differences in growth rate or passage number may influence receptor turnover and these variables have not been studied in the present paper.

Effects of agents that alter 'traffic' to or degradative activities in lysosomes on the turnover of the phosphomannosyl receptor in human fibroblasts

Normal human fibroblasts were pulse-labelled for 90 min with [³⁵S]methionine and then chased for 24

and 48 h in the presence or in the absence of one of the following agents; NH₄Cl (10 mM), leupeptin (20 μM), human spleen β-glucuronidase (20 000 units/ml) or mannose 6-phosphate (2 mM). Phosphomannosyl receptor was immunoprecipitated from the cell lysates, subjected to SDS/polyacrylamide-gel electrophoresis and fluorography and radioactivity in the phosphomannosyl receptor quantified as described in the Materials and methods section. These agents were chosen because of their known effects on the degradative activity of lysosomal proteinases or on the 'traffic' of acid hydrolases to the lysosomes. Leupeptin, an inhibitor of lysosomal thiol proteinases, could be expected to prolong receptor half-life if these enzymes are responsible for receptor degradation (Libby *et al.*, 1980). NH₄Cl, a lysosomotropic amine, raises the lysosomal pH and diverts most newly synthesized lysosomal enzyme precursors into the extracellular medium apparently by impairing receptor re-utilization (Gonzalez-Noriega *et al.*, 1980). Mannose 6-phosphate in the chase medium would block receptor-mediated pinocytosis of any secreted lysosomal enzyme. Saturating amounts of β-glucuronidase in the medium during the chase would continually occupy pinocytosis receptors, and determine to what extent the turnover of the phosphomannosyl receptor is influenced under conditions of maximum internalization of cell-surface receptors. As shown in Fig. 5, all of these agents had only minor influences on phosphomannosyl receptor half-life. The half-life of the phosphomannosyl receptor in controls ranged from 24 to 29 h. The half-life of the receptor was slightly longer in fibroblasts exposed to leupeptin (32 h), slightly shorter in fibroblasts exposed to

NH₄Cl (23 h) and saturating amounts of β -glucuronidase (21 h) and unaffected in cells exposed to mannose 6-phosphate (24 h).

Discussion

The production of antibodies to the phosphomannosyl receptor have enabled us to investigate the biosynthesis and turnover of the phosphomannosyl receptor in human fibroblasts. These studies illustrated the following points. (1) The phosphomannosyl receptor is not synthesized in a detectable precursor form and probably undergoes only oligosaccharide processing. (2) The protein moiety of the phosphomannosyl receptor is degraded only slowly in normal fibroblasts with a half-life of approx. 26 h. (3) Four agents that affect lysosomal enzyme activity or enzyme targeting, leupeptin, NH₄Cl, mannose 6-phosphate and β -glucuronidase had little influence on the turnover of newly synthesized phosphomannosyl receptor. We will discuss each of these points and consider what relevance they have to our understanding of the role of the phosphomannosyl receptor in the intracellular transport of lysosomal enzymes.

These studies found no evidence that the phosphomannosyl receptor is initially synthesized in a precursor form that subsequently undergoes post-translational processing. At the end of a 15 min pulse immunoprecipitable phosphomannosyl receptor co-migrated on SDS/polyacrylamide gels with purified bovine liver phosphomannosyl receptor with an apparent molecular weight of 215 000. Furthermore, in chase periods for up to 3 days we observed no detectable changes in molecular weight of this receptor. These results are similar to those reported for the human transferrin receptor. Omary & Trowbridge (1981) found that the transferrin receptor showed a small but detectable post-translational increase in molecular weight due to the addition of oligosaccharide chains and palmitate. Like the transferrin receptor, the phosphomannosyl receptor is a glycoprotein. Steiner & Rome (1982) found that the pI of the receptor is made more basic by treatment with neuraminidase, presumably due to the removal of sialic acid residues from complex-type oligosaccharide chains. The phosphomannosyl receptor may also contain high-mannose-type oligosaccharide chains as purified bovine liver phosphomannosyl receptors bind tightly to concanavalin A-Sepharose (K. E. Creek & W. S. Sly, unpublished work). Unlike the phosphomannosyl and transferrin receptors, the low-density-lipoprotein receptor has recently been shown to be synthesized as a smaller precursor with an apparent molecular weight of 120 000 that is subsequently converted into a mature receptor with an apparent molecular weight of 160 000 within 15–30 min after

the precursor is synthesized (Tolleshaug *et al.*, 1982).

Using a pulse-chase protocol we determined that the half-life of the phosphomannosyl receptor in human fibroblasts was approx. 26 h. The fact that this receptor is turned over rather slowly agrees with earlier kinetic evidence that it is not degraded after delivery of endocytosed lysosomal enzymes to the lysosome, but is re-utilized (Rome *et al.*, 1979; Gonzalez-Noriega *et al.*, 1980). A similar conclusion was reached for the transferrin receptor by Omary & Trowbridge (1981), who studied its turnover in a human leukaemic T-cell line and found that it is degraded with a half-life of approx. 60 h. Warren & Doyle (1981) reported a turnover rate of approx. 20 h for the asialoglycoprotein receptor present on the cell surface of primary cultures of rat hepatocytes and calculated that during its lifetime each molecule of receptor can deliver about 1000 molecules of ligand to the lysosome to be degraded. Thus, with at least three pinocytosis receptors, several molecules of ligand are probably delivered to their appropriate cellular targets before degradation of the receptor. We found in I-cells, a cell type that produces no endogenous ligand for the phosphomannosyl receptor, that the turnover of the phosphomannosyl receptor was only slightly increased. This result would support the observation that ligand delivery to lysosome does not greatly alter the rate of degradation of the phosphomannosyl receptor.

The finding that the lysosomal thiol- proteinase inhibitor leupeptin only slightly increased the half-life of the phosphomannosyl receptor suggests that these enzymes may play a relatively minor role in the degradation of the receptor. Since NH₄Cl, a lysosomotropic amine that raises the intralysosomal pH and presumably would inhibit lysosomal enzyme activity, also had little effect on phosphomannosyl receptor turnover, this would tend to support the argument that lysosomal acid hydrolases may not be directly involved in receptor degradation. We also found that the presence of saturating amounts of a ligand for the phosphomannosyl receptor, β -glucuronidase, only slightly accelerated receptor turnover. Under these conditions, cell-surface phosphomannosyl receptors were being internalized at maximum rates but must have been largely spared from degradation during receptor-mediated endocytosis of the added ligand. Similar results were reported by Warren & Doyle (1981) for the asialoglycoprotein receptor. Continuous endocytosis of asialo-orosomucoid by rat hepatocyte cultures had no effect on the turnover of the asialoglycoprotein receptor. The finding that the agents we tested all had minor influences, at best, on phosphomannosyl receptor turnover suggest that (1) lysosomal thiol proteinases are probably not important for phosphomannosyl receptor turnover, (2) the delivery of

ligand to the lysosome has little influence on the rate of receptor turnover and (3) the acid pH of the lysosome is not essential for receptor turnover in human fibroblasts.

We would emphasize that our results for this pinocytosis receptor, which mediates enzyme delivery to lysosomes and recycles to be reutilized many times, contrast markedly with studies on the turnover of the acetylcholine receptor in cultured chick myotubes (Libby *et al.*, 1980). The rate of degradation of the acetylcholine receptor, which apparently is not normally recycled (Gardner & Fambrough, 1979), was reduced 2–10-fold by the addition of leupeptin or the lysosomotropic amine chloroquine (Libby *et al.*, 1980). Thus the lysosome probably plays a major role in the turnover of the acetylcholine receptor. The evidence that lysosomes do not play a major role in the turnover of the phosphomannosyl receptor is consistent with more recent evidence that ligands dissociate from pinocytosis receptors in an acidic prelysosomal compartment (Maxfield, 1982). Thus, though the ligands are delivered to lysosomes, the receptors may recycle to the cell surface without actually entering lysosomes.

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