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Antibody to the Phosphomannosyl Receptor Inhibits Recycling of Receptor in Fibroblasts

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The 215-kd phosphomannosyl receptor is involved in the transport of newly synthesized acid hydrolases to lysosomes and also mediates the pinocytosis of lysosomal enzymes by fibroblasts in culture. Recycling of receptors to the sorting sites is an integral part of both these processes. In this report, we describe the inhibition in human fibroblasts of both functions of the phosphomannosyl receptor by a rabbit antiserum to the bovine liver receptor. This inhibition cannot be completely accounted for by inhibition of ligand-receptor interaction. Rather the antibody appears to cross-link receptors and cause a removal of receptors from the sorting sites (plasma membrane and Golgi apparatus) and their accumulation in a compartment from which they do not recycle. Removal of receptors from the recycling pool by antibody is irreversible, and return of receptors requires synthesis of new protein. Degradation of "trapped receptors" is enhanced ($t_{1/2}$ = 7.5 hr), but much more gradual than their removal from the functional receptor pool ($t_{1/2}$ = 30 min).

Key words: receptor inactivation, lysosomal enzyme targeting

The 215-kd phosphomannosyl receptor (PMR), or cation-independent phosphomannosyl receptor, is an integral membrane glycoprotein known to be involved in the targeting of newly synthesized acid hydrolases to lysosomes and in endocytosis of lysosomal enzymes by fibroblasts in culture [1–3]. The receptor has been identified in and purified from many sources including bovine liver [4], Swarm rat chondrosarcoma membranes [5], rat liver [6], and human liver [7]. Antibodies to the receptor have been raised in several laboratories [6–9].

We have used a rabbit antibody to the bovine liver PMR to study the biosynthesis and turnover of the PMR in human fibroblasts and have shown that the receptor has a half-life of 24–29 hr [8]. The receptor is also turned over slowly in monolayers of Chinese hamster ovary cells [10]. The slow turnover of the receptor, together with

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previous kinetic data of ligand internalization, led to the conclusion that, following receptor-mediated endocytosis, the ligand is delivered to lysosomes and the receptor recycles to the plasma membrane to be reutilized [11,12]. Inhibition of recycling by lysosomotropic amines led to the suggestion that pH-dependent release of ligand in an acidic compartment is required for receptors to recycle [12]. The effects of amines on the transport of newly synthesized enzymes suggested that receptor recycling is also an essential feature of transport of newly synthesized acid hydrolases to lysosomes [12,13].

We have previously observed that antibodies to the bovine liver PMR inhibited PMR-mediated endocytosis of β-glucuronidase by human fibroblasts [8]. In this report we further characterize this inhibition and describe how these antibodies also inactivate the targeting of newly synthesized acid hydrolases. We present evidence to suggest that the inactivation of PMR function can be explained by the failure of receptors cross-linked by antibody to recycle to the sites of enzyme sorting (plasma membrane and Golgi apparatus).

MATERIALS AND METHODS

Materials

Goat antirabbit IgG (heavy- and light-chain specific) and fluorescein-conjugated $F(ab^1)_2$ fragment rabbit antigoat IgG (heavy- and light-chain specific) were from Cappel Laboratories (Melvern, PA). [2-^{3}H]-Mannose, (15.8 Ci/mm), [^{35}S]-methionine (953 Ci/mmol), carrier-free $Na^{125}I$ (17 Ci/mg), and EN^{3}HANCE were from New England Nuclear (Boston, MA). β-Nicotinamide adenine dinucleotide, reduced form, and epidermal growth factor (EGF) from mouse submaxillary glands, were from Sigma Chemical Co. (St Louis, MO). Fluorometric substrates were obtained from Research Products International (Elk Grove, IL). Mercuripapain was from Worthington (Freehold, NJ). Iodo-Beads were from Pierce Chemical Co. (Rockford, IL). β-Glucuronidase was purified to homogeneity from human spleen as described previously [14] and an affinity-purified, high-uptake fraction was obtained by chromatography on immobilized phosphomannosyl receptors [15].

Cells and Cell Culture

Normal diploid human fibroblasts (IMR-91), β-glucuronidase-deficient fibroblasts (GM-2784), and Tay-Sachs disease fibroblasts (GM-502) were received from the Human Mutant Cell Repository (Camden, NJ). β-Hexosaminidase-deficient fibroblasts (WH 98/EW) were received from the Repository for Mutant Human Cell Strains (Montreal, Quebec, Canada). Cultures were maintained at 37°C in 5% CO_2 in minimal essential medium MEM, (Earle's Salts, Gibco) containing 15% fetal calf serum, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate.

Preparation of Antibodies

Antibodies to purified bovine liver phosphomannosyl receptor were raised in a rabbit as previously described [8]. An IgG fraction (anti-PMR IgG) was isolated from immune serum by two methods: 1) ammonium sulfate precipitation and chromatography on DEAE-52 [16], and 2) chromatography on protein-A Sepharose CL-4B (Pharmacia, Piscataway, NJ) using 3 M KSCN as eluant, as recommended by the manufacturer.

Anti-PMR Fab fragments were prepared from IgG obtained by the latter method, by papain-digestion [17]. Fab fragments were separated from Fc fragments and from intact IgG molecules by chromatography on protein A Sepharose. Lack of contamination of Fab fragments by IgG was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel [18].

Goat antihuman PMR antibodies were raised by injecting a goat with PMR purified from human liver by the method of Sahagian [9]. Homogeneity of the receptor preparation was confirmed by SDS-PAGE [18]. The antiserum was shown to specifically recognize PMR in human fibroblasts by immunoprecipitation of the receptor from metabolically labeled cells and by immunoblotting of fibroblast membranes (data not shown).

Rabbit antiserum to purified human placental β-hexosaminidase was prepared by Dr. Robert Nicolotti and an IgG fraction was prepared from the antiserum by ammonium sulfate precipitation and chromatography on DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA).

Enzyme and Protein Assays

β-Glucuronidase and β-hexosaminidase activities were determined as described previously [19,20]. Lactate dehydrogenase (LDH) activity was measured spectrophotometrically, as described by Reeves et al [21]. Protein was measured by the method of Lowry et al [22] using bovine serum albumin as standard.

Ligand Iodination

EGF and affinity-purified β-glucuronidase were iodinated with Iodo-Beads, according to the manufacturer's recommendations, to specific radioactivities of 47×10^6 cpm/μg and 39×10^6 cpm/μg, respectively.

Ligand Pinocytosis by Fibroblasts

Pinocytosis of β-glucuronidase was determined in duplicate 35-mm petri dishes containing β-glucuronidase-deficient fibroblasts at confluence (0.2–0.3 mg protein/dish) as previously described [12,23]. Pinocytosis of ^{125}I-EGF was determined in similar dishes by incubating cells with medium containing 10 ng/ml of ^{125}I-EGF, for 30 min at 37°C. Cells were subsequently chilled on ice, rinsed five times with PBS, solubilized in 0.5 ml of 1% sodium deoxycholate, and counted for ^{125}I.

Uptake of β-hexosaminidase was conducted in β-hexosaminidase-deficient Sandhoff disease fibroblasts grown to confluence in 35-mm petri dishes. β-Hexosaminidase (500 units) was added to the cells in 1.0 ml of Eagle's minimal essential medium and incubation was for 2 hr at 37°C. Cell-associated β-hexosaminidase was measured in cells disrupted by freeze-thaw in 0.5 ml of H_2O.

Binding of ^{125}I-β-Glucuronidase

Preparation of total cell membranes and determination of ^{125}I-β-glucuronidase binding to these were as described previously [19]. For determination of cell-surface binding of ^{125}I-β-glucuronidase, confluent monolayers of fibroblasts in 35-mm petri dishes were chilled to 4°C and incubated with 1 ml medium (MEM, 5 mg/ml human serum albumin [HSA], ± 10 mM mannose 6-phosphate (M6-P) containing 250,000 cpm ^{125}I-β-glucuronidase/4,000 units enzyme activity, for 1 hr at 4°C. The monolayers were then washed five times with cold PBS, solubilized in 1 ml 1% DOC, and

counted for ^{125}I. Specific binding was estimated from binding in the presence and absence of M6-P.

Radiolabeling and Immunoprecipitation of β-Hexosaminidase B From Tay-Sachs Disease Fibroblasts

Tay-Sachs disease fibroblasts were grown to confluence in 100-mm petri dishes (Corning). Prior to labeling the cultures were rinsed once with 5.0 ml of low glucose (.28 mM) MEM containing HSA (1 mg/ml), glutamine (2 mM), and pyruvate (0.1 mg/ml) and starved for 30 min in 5.0 ml of this medium at 37°C in a 5% CO_2 atmosphere. The fibroblasts were then labeled for 21 hr in 5.0 ml of the above medium containing [2-^{3}H]mannose, (60 μCi/ml), mannose 6-phosphate (2 mM), and either NH_4Cl (10 mM) or anti-PMR IgG (150 μg/ml).

At the end of the labeling period, the medium was removed and precipitated with 2.5 g of solid $(NH_4)_2SO_4$ for 1 hr at 4°C. The precipitate was collected by centrifugation at 10,000g for 10 min and the pellet was resuspended in 0.5 ml of Dulbecco's phosphate-buffered saline (PBS). Labeled β-hexosaminidase in the medium extract was immunoprecipitated as described below.

Following radiolabeling the cell monolayers were washed twice with 5.0 ml of PBS; the cells were scraped from the dish in PBS with a rubber policeman and pelleted by low-speed centrifugation. The cell pellets were carefully resuspended in PBS and washed a second time. After washing, the cells were resuspended in 0.5 ml of 50 mM Tris, pH 7.0, 10 mM mannose 6-phosphate, 5 mM EDTA, and 10 μg/ml aprotinin and disrupted by three 10-sec bursts with a sonicator (Bronwill Scientific). Sodium deoxycholate was then added to a final concentration of 0.025%; the disrupted cells were placed at 4°C for 30 min, and the membranes were removed by centrifugation at 30,000g for 60 min. The supernatant was used for immunoprecipitation of cell-associated β-hexosaminidase B. Under these conditions greater than 90% of the total cellular β-hexosaminidase is recovered in the cell extract and greater than 90% of the secreted β-hexosaminidase is recovered in the $(NH_4)_2SO_4$ precipitate.

Immunoprecipitation of radiolabeled cellular and secreted β-hexosaminidase B was conducted as follows. [2-^{3}H]Mannose-labeled cell extracts or medium extracts (450 μl) were added to 45 μl of 10% Nonidet P-40, 10% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS) in PBs. Formalin-fixed *Staphylococcus aureus* (50 μl of a 10% v/v suspension) was added and pelleted after 30 min at room temperature. The supernatants were transferred to new tubes and antigen-antibody complexes were formed by the addition of 25 μl of rabbit antihuman placental β-hexosaminidase antibodies (45 μg of IgG). After 2 hr at room temperature, 50 μl of formalin-fixed *S. aureus* was added, and immune complexes were collected 30 min later by centrifugation for 1 min in a Beckman microfuge. These complexes were resuspended in 1.0 ml of 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.05% SDS in PBS, transferred to a new tube, and washed two more times with 0.5 ml of this detergent-containing buffer, once with this buffer containing 350 mM added NaCl, and finally once with 0.5 ml of PBS. The immunoprecipitates were released from the *S aureus* by boiling for 5 min in 100 μl of sample buffer (75 mM Tris; pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 5% glycerol, 0.0015% bromophenol blue). Polyacrylamide gel electrophoresis was conducted under reducing conditions in 10% polyacrylamide gels containing 0.1% (w/v) SDS as described by Laemmli [18]. Bio-Rad (Richmond, CA) low molecular weight standards were visualized by staining with Coomassie Blue.

Gels were subsequently processed for fluorography in EN^{3}HANCE and exposed to Kodak XSAR-5 film at −70°C for 9 days.

Immunofluorescence

Subconfluent fibroblasts on tissue culture chamber slides were fixed and permeabilized in 100% methanol at 4°C for 10 min. They were incubated for 1 hr, at room temperature, with goat antihuman liver PMR serum (diluted 1:30 in PBS containing 1% normal rabbit serum and 0.2% gelatin) followed by fluorescein-conjugated rabbit F (ab^1)$_2$ antigoat IgG (diluted 1:20 in the same buffer) and examined and photographed with a Leitz orthoplan fluorescent microscope with a filter suitable for green fluorescence (520 nm).

In control experiments, it was shown that binding of rabbit antibovine liver PMR by fibroblasts did not interfere with subsequent detection of PMR in these cells by the goat anti-PMR serum.

RESULTS

Figure 1A shows the inhibition of β-glucuronidase uptake with increasing concentrations of antibovine liver-PMR serum in the medium. At an antiserum concentration of 40 μl/ml, β-glucuronidase uptake is less than 20% that of control, non-antibody-treated cells. Preimmune serum showed no inhibitory effect on β-glucuronidase uptake (data not shown). Figure 1A also shows that the uptake of ^{125}I-EGF, another molecule internalized by receptor-mediated endocytosis, is unaffected. Thus, the inhibitory effect on pinocytosis is specific for PMR-mediated pinocytosis.

In order to determine whether inhibition of enzyme pinocytosis was secondary to blocking of the ligand binding site of the PMR by the antibody, we measured the effect of anti-PMR antiserum on 4°C cell-surface binding, using ^{125}I-β-glucuronidase. As shown in Figure 1B, anti-PMR serum interfered with binding to a maximum of 30%. This interference, however, was barely, if at all, greater than that seen with preimmune serum and could not fully account for the observed inhibition of endocytosis. The inhibition of binding by preimmune serum may be due to competition from other mannose-6-P (M6-P)-containing acid hydrolases which are released from platelets during the clotting process in the preparation of rabbit serum.

The almost complete inhibition of ligand internalization, in the absence of a comparably large effect on ligand binding, suggested the possibility of antibody-induced removal of receptors from the cell surface. To test this, we incubated cells at 37°C with a 50 μl/ml concentration of anti-PMR serum for various time intervals and measured the cell-surface enzyme binding activity and enzyme pinocytosis (Fig. 2). Both activities decreased in a similar, time-dependent manner, with the half-life of both processes being 25–30 min. This is consistent with loss of pinocytosis activity being a consequence of disappearance of receptors from the cell surface.

Several studies with other ligands and their receptors have shown a decrease in cell-surface receptor number on treatment of cells with antireceptor antibodies [24–30]. In some cases, this decrease has been ascribed to antibody-induced cross-linking of receptors and their subsequent failure to recycle to the surface [24–27]. To see if antibody-induced cross-linking of PMR might be a factor in the inactivation of pinocytosis of β-glucuronidase, we prepared anti-PMR Fab fragments by papain digestion of anti-PMR IgG and examined their effects on β-glucuronidase uptake.

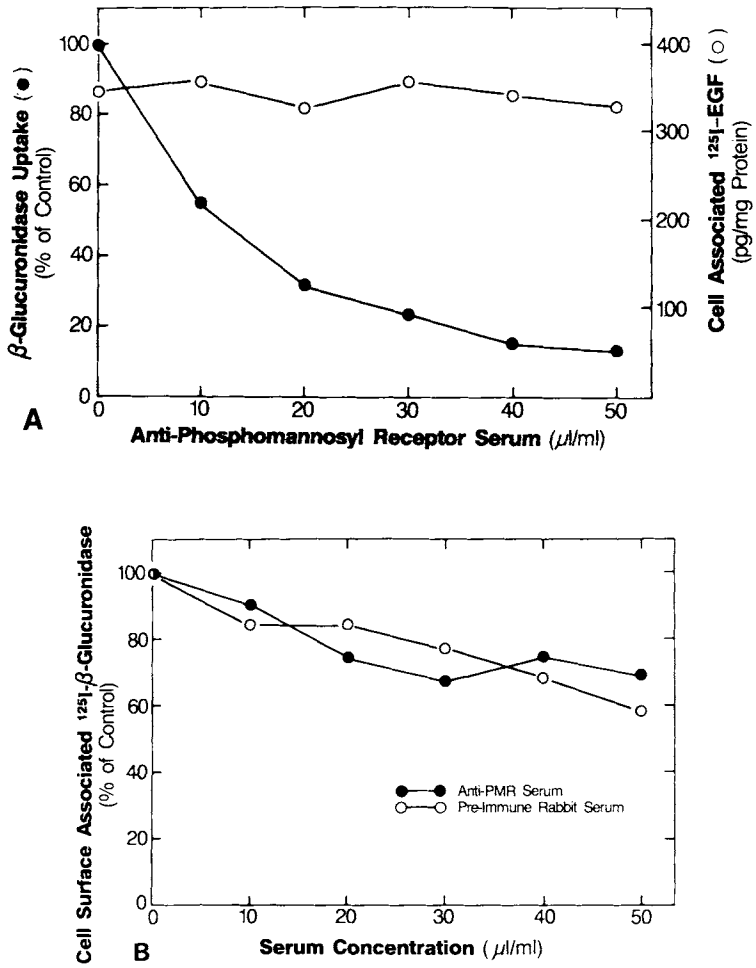

Fig. 1. The effect of antibovine liver-PMR serum on pinocytosis of β-glucuronidase and on cell-surface binding of ^{125}I-β-glucuronidase by human fibroblasts. **A:** Confluent monolayers of β-glucuronidase-deficient fibroblasts, in 35-mm dishes, were incubated for 2 hr, at 37°C, with 1.0 ml of Eagle MEM containing increasing concentrations of anti-PMR serum. The cells were then washed with PBS and pinocytosis of β-glucuronidase (●) and of ^{125}I-EGF (○) were measured. Enzyme pinocytosis refers to M6-P-inhibitible enzyme uptake determined by incubating cells with 4,000 units/ml of human spleen β-glucuronidase for 2 hr at 37°C in the presence or absence of 2 mM M6-P. Enzyme was assayed fluorometrically in cell lysates. Pinocytosis of ^{125}I-EGF was determined following incubation of cells for 30 min at 37°C with 10 μg/ml of ^{125}I-EGF with or without excess unlabeled EGF. Cell lysates were counted for ^{125}I. **B:** Similar monolayers of fibroblasts were incubated for 1 hr at 4°C with 1 ml of Eagle MEM containing the indicated concentrations of normal rabbit serum (○) or anti-PMR serum (●). Following removal of the medium, the cells were washed once with 2 ml of ice-cold medium and M6-P-specific cell-surface binding of ^{125}I-β-glucuronidase was measured. Cells were chilled to 4°C and incubated with ^{125}I-β-glucuronidase (250,000 cpm/4,000 units enzyme activity) for 1 hr at 4°C in the presence or absence of 10 mM M6-P. Cell lysates were counted for ^{125}I. Uptake of β-glucuronidase and ^{125}I-β-glucuronidase binding are expressed as % of control, nonantibody-treated cells, while ^{125}I-EGF uptake is represented as pg/mg protein.

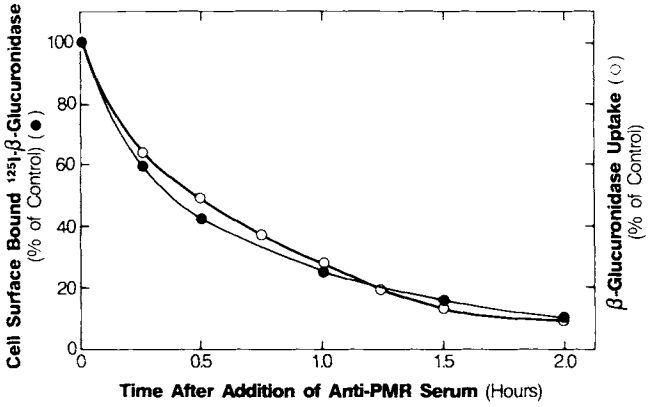

Fig. 2. Kinetics of inactivation of pinocytosis and of loss of cell-surface binding activity. Confluent monolayers of β-glucuronidase-deficient fibroblasts were incubated at 37°C with 1 ml of medium containing 50 μl of anti-PMR serum. At the indicated times, the medium was removed, cells were washed with MEM, and pinocytosis of β-glucuronidase (○) or cell-surface binding of ^{125}I-β-glucuronidase (●) was measured, as described in the legend to Figure 1. Activities are expressed as % of those of control, nonantibody-treated cells.

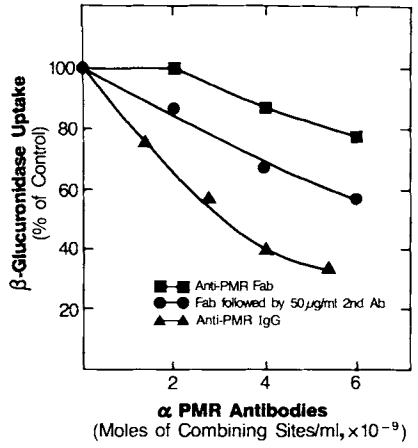

Fig. 3. Cross-linking of phosphomannosyl receptors is of importance in inactivation of β-glucuronidase pinocytosis. Confluent monolayers of β-glucuronidase-deficient fibroblasts, in 35-mm petri dishes, were incubated for 2 hr, at 37°C, with 1 ml of medium containing increasing concentrations of anti-PMR IgG (▲) or of anti-PMR Fab fragments (■). Following exposure to anti-PMR Fab fragments, one set of dishes was incubated for a further 2 hr at 37°C with (50 μg/ml) goat antirabbit IgG (●). The monolayers were then washed with MEM and pinocytosis of β-glucuronidase was measured, as described in the legend to Figure 1. Pinocytotic activity is expressed as % of control nonantibody-treated cells.

Figure 3 shows that anti-PMR Fab fragments inhibited endocytosis by about 25% while intact IgG produced a 65% inhibition. The effectiveness of the Fab fragments could be increased, however, by the addition of a cross-linking second antibody (goat antirabbit IgG, specific for heavy and light chains) to cells previously incubated with Fab fragments (Fig. 3). The second antibody alone had no effect on pinocytosis. These results suggest that cross-linking of receptors by the anti-PMR antibody may be of importance but not necessary in the antibody-induced inactivation of pinocytosis.

We next examined the recovery of cells from antibody treatment. Recovery of pinocytotic activity was slow compared to its inactivation; 48 hr was required for β-glucuronidase uptake of antibody-treated cells to equal 80% that of controls (Fig. 4). This rate of recovery was similar to the turnover rate of PMR in human fibroblasts ($t_{1/2}$ = 24–29 hr) [8] and suggested that synthesis of new receptors is required for recovery of pinocytosis. To test this, antibody-treated cells were allowed to recover in the presence or absence of 100 μM cycloheximide for 24 hr, and cell-surface binding activity was then measured with ^{125}I-β-glucuronidase. In the absence of cycloheximide, antibody-treated cells recovered 72% of the binding of control, nonantibody-treated cells. Cycloheximide-treated cells only reached 25% of normal binding (Fig. 4). (Cells which had not been preincubated with antiserum, but were incubated for 24 hr in the presence of 100 μM cycloheximide, had 63% of control binding activity, consistent with the 24–29-hr half-life of the receptor estimated from metabolic labeling experiments [8].)

The above results suggested that antibody-induced disappearance of cell-surface PMR activity was essentially irreversible in the absence of new protein synthesis. This is in contrast to the chloroquine-induced loss of cell-surface receptors, which is rapidly reversed on removal of the inhibitor [12]. Trypsin treatment of cells also has been reported to cause a loss of cell-surface binding activity which is quite rapidly reversed, at least partially, by externalization of receptors from the intracellular pool [11]. The inability of antibody-treated cells to recover in such a rapid manner suggests that, on prolonged exposure to antibody, the intracellular receptor pool also becomes inactivated by the anti-PMR antibodies. Sahagian has previously shown that intracel-

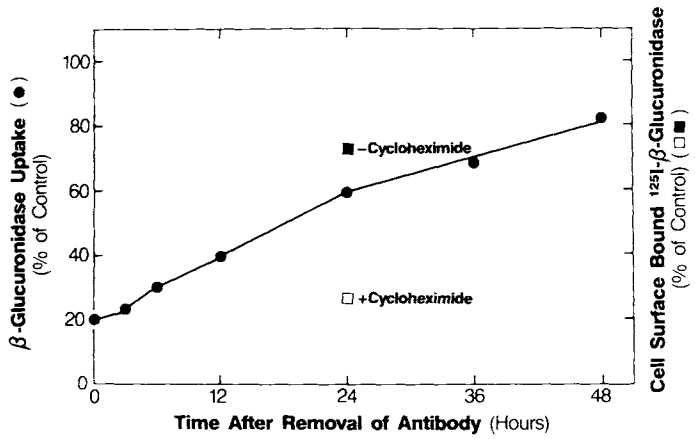

Fig. 4. Recovery of β-glucuronidase pinocytosis and of cell-surface binding activity following removal of anti-PMR serum. Confluent monolayers of β-glucuronidase-deficient fibroblasts, in 35-mm dishes, were incubated with 1 ml of medium containing 50 μl of anti-PMR serum for 2 hr at 37°C. This medium was then removed and cells were incubated at 37°C with nonantibody-containing medium. At the indicated times, β-glucuronidase pinocytotic activity (●) was measured as described in the legend to Figure 1. The effect of cycloheximide on recovery of cell-surface binding activity was examined by incubating cells with medium containing anti-PMR serum for 2 hr, as above. Antibody containing medium was then replaced by fresh medium and cells were allowed to recover in the presence (□) or absence (■) of 100 μM cycloheximide. At the end of the recovery period, cell-surface binding of ^{125}I-β-glucuronidase was measured, as described in the legend to Figure 1. Pinocytotic and binding activities are expressed as % of control plates not exposed to anti-PMR serum.

lular PMR is accessible to exogenously added antibody [31]. It was of interest, therefore, to examine the effect of anti-PMR antibody on the function of the intracellular PMR.

Impairment of intracellular PMR function, by chloroquine or NH_4Cl, results in the enhanced secretion of precursor forms of acid hydrolases [12, 13]. Exposure to anti-PMR antiserum had similar effects. The effects of increasing concentrations of anti-PMR IgG and of normal rabbit IgG on the secretion of β-hexosaminidase by fibroblasts over a 24-hr period are shown in Figure 5. At a concentration of 150 μg/ml of anti-PMR IgG, the level of β-hexosaminidase in the medium was 71% that of cells incubated for 24 hr in the presence of 10 mM NH_4Cl (data not shown). This antibody-induced secretion was not due to nonspecific cell damage or lysis, as indicated by the lack of effects of anti-PMR IgG on extracellular levels of the cytosolic enzyme lactate dehydrogenase (Fig. 5).

The β-hexosaminidase secreted in response to antibody treatment was a "high-uptake" form, as has been previously reported for enzyme precursor secreted in the presence of NH_4Cl [12]. This was indicated by the fact that the rate of endocytosis of this enzyme was equal to that of β-hexosaminidase secreted by NH_4Cl-treated fibroblasts (Table I). Figure 6 shows that β-hexosaminidase B secreted by Tay-Sachs disease fibroblasts during a 21-hr exposure to 150 μg/ml of anti-PMR IgG was the 63-kd precursor form and not the 29-kd processed form which predominates in lysosomes [13]. This indicates that the secreted enzyme was newly synthesized and not enzyme removed from the lysosome. Anti-PMR antibodies, therefore, in addition to inactivating PMR-mediated pinocytosis, disrupt the intracellular sorting of newly synthesized acid hydrolases and enhance their secretion.

It has previously been reported that in cells treated with tunicamycin or chloroquine, the disruption of trafficking of acid hydrolases is correlated with a redistribution of PMR away from its steady-state location [32]. We examined the distribution of PMR in fibroblasts treated with anti-PMR serum, by indirect immunofluorescence.

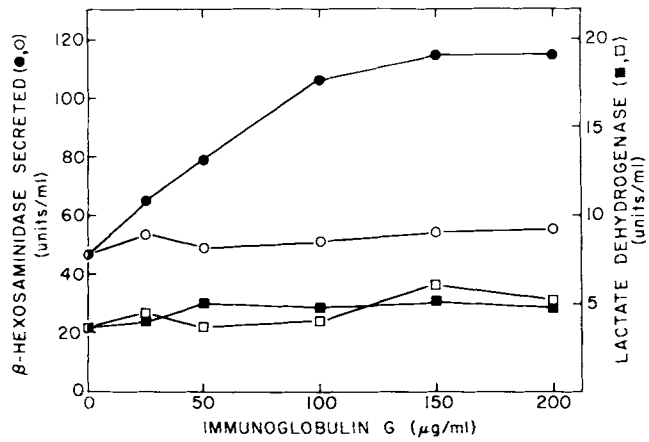

Fig. 5. Effect of anti-PMR IgG on secretion of β-hexosaminidase by human fibroblasts. Confluent 35-mm petri dishes of normal human fibroblasts were incubated for 24 hr in 2.0 ml of Waymouth medium containing 1 mg/ml human serum albumin, 2 mM glutamine, and the indicated concentrations of normal rabbit IgG (○,□), or anti-PMR IgG (●,■). The levels of β-hexosaminidase (○,●) and lactate dehydrogenase (□,■) activities in the media were then determined.

TABLE I. Pinocytosis of β-Hexosaminidase Secreted in Response to Anti-PMR IgG or NH_4Cl*

Treatment	Pinocytosis of β-hexosaminidase (units/mg protein/hr)
Anti-PMR IgG (150 μg/ml)	9.5
NH_4Cl (10 mM)	9.1

*Confluent 100-mm petri dishes of normal human fibroblasts were incubated in 5.0 ml of Waymouth medium containing 1 mg/ml human serum albumin, 2 mM glutamine, 2 mM mannose-6-phosphate, and either 150 μg/ml anti-PMR IgG or 10 mM NH_4Cl. After 24 hr the medium was removed and the anti-PMR IgG was removed by affinity chromatography on a 0.5 ml column of Protein A-Sepharose (Pharmacia). The medium was dialyzed against PBS to remove mannose-6-phosphate and NH_4Cl and then concentrated tenfold by ultrafiltration using a PM 10 membrane filter (Amicon). Uptake of β-hexosaminidase was measured as described in Materials and Methods.

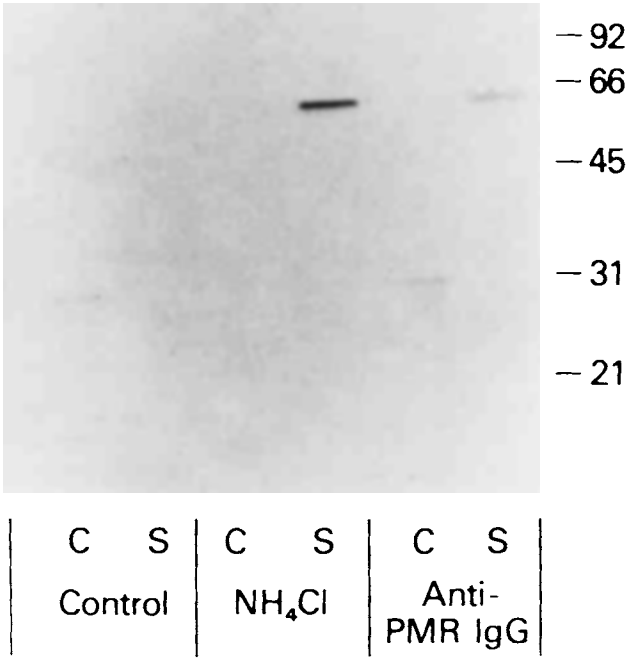

Fig. 6. Anti-PMR IgG causes secretion of precursor β-hexosaminidase B by Tay-Sachs disease fibroblasts. Confluent Tay-Sachs disease fibroblasts were continuously labeled for 21 hr with [2-^{3}H]mannose (60 μCi/ml) in the absence (control) or presence of 10 mM NH_4Cl or 150 μg/ml anti-PMR IgG. Cellular (C) and secreted (S) β-hexosaminidase B was immunoprecipitated and the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and fluorography, as described in Materials and Methods. The position of the following molecular weight standards are indicated: phosphorylase b, 92,500; bovine serum albumin 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500. Bio-Rad, Richmond, CA.

Cells which had been preincubated with preimmune rabbit serum for 2 or 8 hr showed a bright, predominantly juxtanuclear fluorescence pattern (Fig. 7A,C), consistent with previously reported localization of the PMR in fibroblasts to the Golgi cisternae, associated coated vesicles, and larger organelles [33]. The cells preincubated with antibovine liver-PMR serum for 2 hr, however, showed a different staining pattern. Staining was much less intense in the juxtanuclear area and was more peripherally located, in a largely punctate pattern (Fig. 7B). Thus, there is an antibody-induced redistribution of receptor which correlates with functional impairment of the PMR's ability to target newly synthesized acid hydrolases to lysosomes. Following pretreatment of cells with anti-PMR serum for 8 hr, the cells did not show any significant staining for PMR (Fig. 7D), suggesting antibody-induced receptor degradation.

The fact that the antibovine liver-PMR antibody inhibited ligand binding by the receptor to only a small extent allowed us to examine the binding activity of the receptor after the antibody-induced redistribution from its normal cell surface and intracellular locations. Cells were incubated with antiserum for various times, following which total cell membranes were prepared and tested for binding of ^{125}I-β-glucuronidase (Fig. 8). Anti-PMR-treated cells showed a gradual decrease in total binding activity (cell surface and intracellular), with a half-life of 7.5 hr. Thus, although loss of enzyme pinocytosis and receptor redistribution occurs rapidly (within 2 hr), the ligand binding property of the internal pool of receptors is much more slowly inactivated. These observations are consistent with the idea that antibody

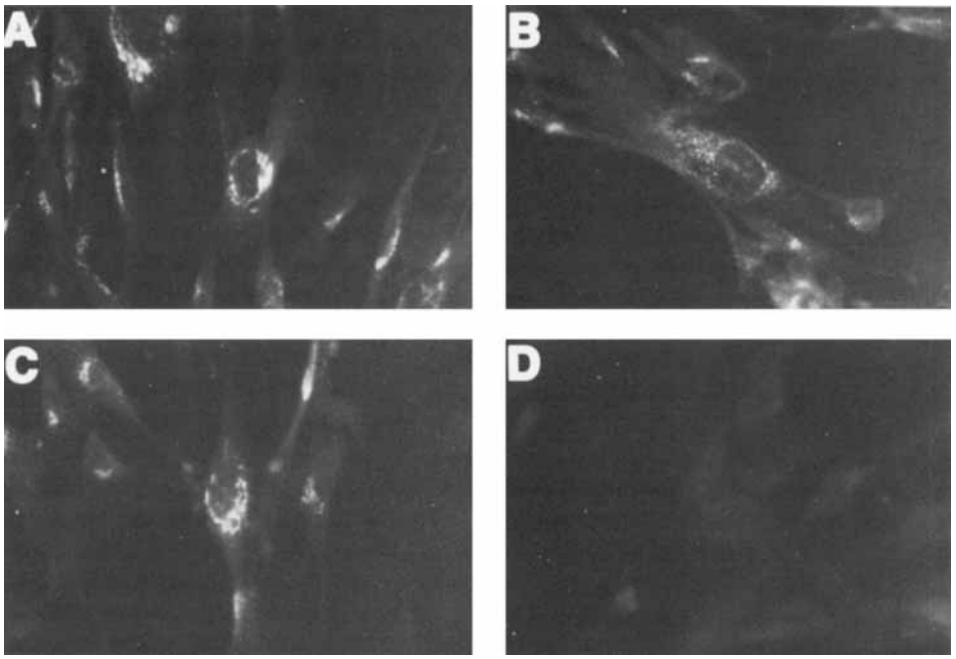

Fig. 7. Anti-PMR serum causes a redistribution of intracellular PMR. Subconfluent monolayers of human fibroblasts were incubated for 2 hr (**A,B**) or 8 hr (**C,D**) at 37°C, with medium containing a 1:20 dilution of preimmune rabbit serum (A,C) or of antibovine liver PMR serum (B,D). Cells were fixed in methanol and stained for PMR by using goat antihuman liver PMR followed by fluorescein-conjugated $F(ab^1)_2$ rabbit antigoat IgG, as described in Materials and Methods.

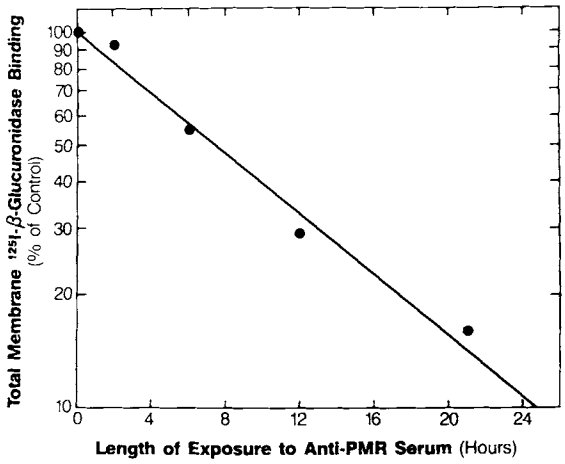

Fig. 8. Kinetics of loss of total membrane binding activity. Confluent monolayers of fibroblasts were incubated with 5 ml of medium containing 250 μl of antibovine liver-PMR serum. At the indicated times, cells were harvested, total membranes were prepared, and binding of ^{125}I-β-glucuronidase was measured by incubating membranes with ^{125}I-β-glucuronidase (250,000 cpm/4,000 units enzyme activity) for 1 hr at 4°C in the presence or absence of 10 mM M6-P. Binding activity is expressed as % of control, nonantibody-treated cells.

treatment inhibits the functioning of the PMR not by blocking binding, but rather by interfering with its recycling to the sites of enzyme sorting (ie, the cell surface for enzyme pinocytosis and the Golgi apparatus for sorting of newly synthesized acid hydrolases).

DISCUSSION

Studies of several cell-surface receptors, including the low-density lipoprotein (LDL) receptor [24], the epidermal growth factor (EGF) receptor [25], transferrin receptor [28], and the insulin receptor [29,30], have shown a reduction in cell-surface receptor number in cells exposed to the respective antibodies. In the case of the LDL [24] and EGF [25] receptors, this reduction has been attributed to the prevention of receptor recycling by antibody-induced cross-linking of receptors. This interpretation was supported by the fact that the effect was produced by polyclonal IgG but not by Fab fragments or by monoclonal antibodies. The loss of cell-surface transferrin-receptor induced by the monoclonal antibody, OKT9, has been suggested to be due to an increased rate of departure of receptors from the recycling pool and not to an inhibition of recycling [28].

Antibody-induced cross-linking of receptors appears to be of importance in the inactivation of endocytosis by anti-PMR antibodies, though this may not be the sole mechanism of inactivation. Monovalent anti-PMR Fab fragments were less inhibitory than intact IgG. The effectiveness of the Fab fragments could be increased by incubating the cells with a second cross-linking antibody which, by itself, had no effect on pinocytosis. It is interesting that anti-PMR IgG was not as effective an inhibitor of endocytosis as the unfractionated antiserum. It may be that the anti-PMR serum contains a population of polyvalent IgM molecules which contribute to the inactivation process and which are lost on preparation of IgG.

While the antibody-induced inhibition of endocytosis and loss of cell-surface receptors are very rapid processes, with half-lives of 25–30 min, full recovery of these activities is much slower and requires synthesis of new receptors. The cell-surface PMR of fibroblasts constitutes only about 10–20% of total cell-associated PMR, the remainder being intracellular [34]. The requirement for synthesis of new receptors for recovery of cell-surface binding activity following antibody treatment suggested that the intracellular PMR pool was also inactivated by the antibody and unable to replace the inactivated cell-surface receptors.

Further evidence that the intracellular pool of enzyme receptors was accessible to and inactivated by anti-PMR antibody was provided by studies of enzyme secretion. Antibody-treated cells showed enhanced secretion of β-hexosaminidase, which was quantitatively similar to the enhanced secretion produced by NH_4Cl, and qualitatively similar in that the secreted form was the precursor enzyme and not the processed mature form.

Whether the intracellular receptors which participate in sorting newly synthesized acid hydrolases are inactivated by antibody at the cell surface, which would imply that they were in equilibrium with the cell-surface receptors which mediate enzyme pinocytosis, or are inactivated by antibody that is internalized by fluid phase endocytosis, is unclear. What is clear is that intracellular PMR is accessible to and functionally inactivated by the anti-PMR antibody. Sahagian has also reported the accessibility of intracellular PMR to exogenously added antibodies and the equilibration of cell-surface and intracellular pools of receptor [31].

We have previously proposed that PMR function, both in enzyme pinocytosis and in sorting newly synthesized acid hydrolases, is dependent on receptor reutilization or recycling [12]. We have suggested that the impairment of both processes by amines is explainable by the ability of these agents to inhibit receptor recycling. Similarly, we propose that the inhibition of both of these processes by antibody which does not block ligand binding is due to impairment of receptor recycling. However, unlike impairment by amines, which is secondary to inhibition of pH-dependent dissociation of ligand from receptor, and which is freely reversible on removal of amines, inactivation of receptors by antibodies is essentially irreversible. There appear to be two components to the inactivation—a removal of functional receptor from the recycling pool, which is rapid and which we attribute to receptor cross-linking, and a slower process of somewhat accelerated degradation of antibody-inactivated receptor, the mechanism of which is not yet clear. The morphological equivalent of the early component of the inactivation is redistribution of most of the receptors from perinuclear (presumably Golgi) distribution to more peripheral vesicles. The morphological equivalent of the second component of the inactivation is disappearance of the immunofluorescent PMR signal.

Prior studies have suggested that most of the normal turnover of the PMR in fibroblasts does not involve degradation in lysosomes, since turnover was nearly normal in I-cell fibroblasts and was not markedly inhibited by NH_4Cl or leupeptin [8]. We are also unable to demonstrate any effect of leupeptin or NH_4Cl on the rate of loss of binding activity in antibody-treated cells (data not shown). Thus, the site of enhanced turnover of the PMR is not clear.

Antibody-induced PMR deficiency in human fibroblasts has previously been reported by von Figura and co-workers [7,35]. This deficiency was also characterized by impairment of endocytosis of exogenous ligands and of targeting of newly synthe-

sized acid hydrolases. However, the mechanism of PMR inactivation appears to be different. This may be due to differences in the antibodies used. The antihuman liver-PMR antibodies used by von Figura's group blocked the PMR binding site, and inhibition of pinocytosis and targeting was attributed to this property. Furthermore, these authors concluded that neither polyvalent nor monovalent antibodies prevented receptor cycling between the cell surface and the intracellular compartments. This interpretation contrasts with the conclusions we draw from the results reported here. With our antibody, inactivation cannot be readily explained by interference with ligand binding. We concluded that cross-linking of receptors by antibody is of importance in the inactivation of receptor by this antibody, and that the inactivation results from the inability of cross-linked receptor which is still able to bind ligand, to recycle to the site of sorting.

Several conditions have now been established in which function of phosphomannosyl receptors is impaired. These include conditions where the ligand is defective, as in I-cell fibroblasts [36] or in normal cells treated with tunicamycin [37], and conditions where ligand-receptor dissociation is prevented, as in NH_4Cl- or chloroquine-treated cells [12,13], or in mutant cells which are defective in the ability to acidify their endosomes [38]. The antibody-induced impairment of PMR function reported in this manuscript and that observed by von Figura and co-workers [7,35] represent systems in which the disruption of receptor function is due to direct effects on the receptor itself, in one case blocking the ligand binding to receptor, and in the other, blocking receptor recycling. The ability to perturb the function of the PMR with these reagents may be of use in studying the pathways by which acid hydrolases are sorted.

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