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Properties of the Syrian hamster phosphomannosyl receptor: An aggregate of low molecular weight proteins

(phosphomannosyl receptor dissociation/immunoblotting/tissue-related components)

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ABSTRACT **Phosphomannosyl receptor (PMR) isolated from Syrian hamster liver was purified to apparent homogeneity by affinity chromatography and one-dimensional PAGE. On one-dimensional PAGE, the receptor migrated with a M_r ≈215,000 as detected by a silver-staining reagent or by immunoblotting [utilizing antiPMR generated against purified hamster PMR (M_r 215,000) sequentially purified by affinity chromatography and NaDodSO$_4$/PAGE]. On two-dimensional PAGE the receptor was partially dissociated into low-molecular weight components. The protein distribution on immunoblots of two-dimensional gels of hamster liver homogenates was nearly identical to that observed for purified hamster liver PMR. When liver homogenates were subjected to one-dimensional PAGE and immunoblotted under nonreducing conditions, an intensely labeled band that migrated with an apparent M_r of 43,000–49,000 was observed; under reducing conditions a single band with a M_r of 49,000 was observed. The low molecular weight compound was present in the soluble 130,000 × *g* supernatant but not in the particulate fraction of liver homogenates. An immunoreactive component of similar molecular weight was also present in hamster serum and plasma. These results suggest that PMR is either an aggregate comprised of small molecular weight components or, alternatively, that a number of small molecular weight components are tightly associated with PMR.**

The phosphomannosyl receptor (PMR) is presumed to play a major role in the delivery of newly synthesized and extracellular lysosomal enzymes to lysosomes. Mannose 6-phosphate residues (Man-6-*P*) present on the oligosaccharides of acid hydrolases bind to the receptor, and the bound acid hydrolases are transported into a prelysosomal compartment and finally into lysosomes (1–4).

The PMR has been demonstrated in bovine liver (5), Swarm rat chondrosarcoma chondrocytes (6), rat hepatocytes (5, 7), CHO cells (5, 8), mouse cell lines (9), rabbit alveolar macrophages (10), human fibroblasts (5, 11, 12), rat tissues (13), and rat liver and calf brain coated vesicle preparations (14, 15). Originally isolated from bovine hepatocyte plasma membranes (5), PMR was subsequently prepared by affinity chromatography from bovine liver (16), Swarm rat chondrosarcoma (6), monkey brain (17), and rat spleen (2). Each preparation, with the exception of monkey brain, when subjected to one-dimension (1D) PAGE under reducing conditions exhibited an apparent M_r of 215,000.

Mitchell *et al.* (18) showed that bovine liver membrane preparations extracted with Zwittergent 3-14 yielded in addition to PMR (M_r 215,000), a second protein migrating with an apparent M_r of 57,000. The same low molecular weight component was also obtained from PMR (M_r 215,000) preparations which were incubated at 4°C for long periods of time in the presence of Zwittergent and in the absence of Man-6-*P*. Other investigators have noted low molecular weight forms related to the PMR in receptor preparations (5, 17) and in immunoprecipitates of cells labeled with radioactive precursors (19).

This communication presents evidence (*i*) that hamster PMR is partially dissociated by two-dimensional (2D) PAGE, (*ii*) that multiple low molecular weight components, immunochemically related to PMR, are present in hamster liver homogenates, (*iii*) that the low molecular weight compound(s) in liver homogenates are associated exclusively with the soluble fraction (130,000 × *g* supernatant), and (*iv*) that immunoreactive compounds of similar molecular weights are also present in hamster serum and plasma.

MATERIALS AND METHODS

Polyacrylamide gel electrophoresis reagents and supplies were obtained from Bio-Rad, Serva (Heidelberg, FRG) Bethesda Research Laboratories. High and low molecular weight standards, 4-chloro-1-naphthol, and the Bradford protein assay reagent were obtained from Bio-Rad. Nitrocellulose sheets BA 85 (0.45 μm) were obtained from Schleicher & Schuell, ^{125}I was from Amersham, and protein A, Man-6-*P*, Triton X-100, Nonidet P-40, and goat anti-rabbit peroxidase-conjugated IgG were obtained from Sigma. Iodobeads were obtained from Pierce, Ampholines (pH 3.5–10) from LKB, PD-10 columns from Pharmacia, and x-ray film XR-5 from Eastman Kodak. Adult Syrian hamsters were obtained from the Charles River Breeding Laboratories.

Isolation of the PMR. PMR was isolated by the method of Sahagian *et al.* (16) from the livers of exsanguinated Syrian hamsters and was stored at 4°C in the presence of 5 mM Man-6-*P* until used.

PAGE. 1D-PAGE was performed with 7.5% gels according to procedure of Laemmli (20) in the presence and absence of 5% (vol/vol) 2-mercaptoethanol. 2D-PAGE was conducted by modification of the procedure of O'Farrell (21). Samples subjected to 2D-PAGE were diluted (1:1) with a solubilization solution containing 9 M urea, 2% (vol/vol) Nonidet P-40, 2% (vol/vol) 2-mercaptoethanol, and 2% (vol/vol) Ampholine (pH 3.5–10). The solubilized samples were applied to isoelectric focusing gels (first dimension) using the conditions of Anderson and Anderson (22). After focusing (15 hr at 1200 V and then 2 hr at 1500 V), gels were extruded from the tubes and equilibrated (21). The NaDodSO$_4$-containing slab gels used for electrophoresis in the second dimension were

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Abbreviations: PMR, phosphomannosyl receptor; 1D-PAGE, one-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; Man-6-*P*, mannose 6-phosphate.

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prepared with an 11.25–13.75% polyacrylamide gradient (23). After electrophoresis, gels not used for immunoblotting were stained with Coomassie blue and then by the silver-staining method as described by Merril *et al.* (24).

Preparation of Antibody to Reduced, Denatured PMR. Affinity-purified Syrian-hamster PMR was subjected to 1D-PAGE under reducing conditions, and the gels were stained with Coomassie blue. The material migrating with an apparent M_r of 215,000 was cut from the gels and electroeluted into 0.63 mM Tris·HCl, 48 mM glycine, and 0.1% $NaDodSO_4$ (pH 8.3). Rabbits were challenged initially with multiple intradermal injections of a suspension of electroeluted PMR (100 μg) in Freund's complete adjuvant. At three subsequent times two weeks apart, the rabbits were injected in the same manner with PMR (50 μg) suspended in Freund's incomplete adjuvant.

Immunoblotting. Protein blotting was conducted for 16 hr at 60 V from 1D-PAGE and 2D-PAGE gels, as described by Towbin *et al.* (25). The blots were incubated for 5 hr with appropriately diluted antiserum, washed with Tris-buffered saline, pH 7.4, containing 0.05% Nonidet P-40 and the protein–antibody complexes detected by (*i*) incubation with ^{125}I-labeled protein A (followed by washing with the same buffer) and autoradiography, or (*ii*) by binding peroxidase-labeled goat anti-rabbit IgG. The peroxidase was detected by incubation with 4-chloro-1-naphthol (26).

Protein A was labeled with ^{125}I by using Iodobeads as described by the manufacturer. The labeled product (specific activity, ≈10 μCi/μg; 1 Ci = 37 GBq) was separated from ^{125}I by gel filtration with a PD-10 column.

Protein Determination. The protein content of purified PMR preparations containing Triton X-100 was determined by the method of Peterson (27). In the absence of detergent, protein content was determined by the method of Bradford (28).

RESULTS

Dissociation of Purified Hamster PMR into Low Molecular Weight Components. When PMR, isolated by the procedure of Sahagian (16), was applied to one-dimensional gels under reducing conditions, a major band with an apparent M_r of 215,000 was detected by silver-staining. When the same preparation was subjected to 1D-PAGE under nonreducing conditions, three bands were detected, a major band with an apparent M_r of 190,000 and two additional bands with M_r values >215,000 (Fig. 1*A*). Similar results were obtained when larger amounts (10 μg) of reduced and nonreduced PMR were electrophoresed on one-dimensional gels, and the gels were stained with Coomassie blue. These results are similar to those found for bovine PMR (5, 16). However, when an aliquot of the same PMR preparation was subjected to 2D-PAGE and stained with silver reagent, multiple spots were obtained (Fig. 1*B*). The detection of multiple components of differing pI values and M_r values (50,000–70,000) (Fig. 1*B*, areas a–d) was not anticipated. These results differ from those obtained with bovine liver (3) and Swarm rat chondrosarcoma chondrocytes (6). When bovine liver PMR was subjected to one-dimensional isoelectric focusing, three proteins were detected (3). Swarm rat chondrosarcoma PMR when subjected to 2D-PAGE separated into two isoelectric forms. Treatment of hamster PMR with 4 M urea or 2% (vol/vol) Nonidet P-40, reduction and alkylation, incubation with Ampholine (pH 3.5–10), alkaline phosphatase, or precipitation with ethanol (to remove Triton X-100) failed to promote dissociation of PMR as determined by 1D-PAGE.

Demonstration of Low Molecular Weight Proteins in Hamster Liver Homogenates That React with Anti-PMR Antiserum. Freshly prepared hamster liver homogenates were subjected to 1D-PAGE under nonreducing conditions, and the gels were immunoblotted [Fig. 2*A* (−)]. Several bands were detected, a broad, intense-staining band that failed to penetrate the 7.5% gel, two minor bands with apparent M_r values of 210,000 and 200,000, respectively, [similar in migration to compounds seen on gels containing purified hamster PMR (Fig. 1*A*)], and a major, intense-staining, immunoreactive band that migrated in a M_r range of 43,000–49,000. The intensity of the band noted at the interface between the stacking and running gels varied greatly from preparation to preparation and may represent binding of antibody to precipitated or aggregated PMR (or immunochemically related compounds) of sufficient size to prevent penetration into the gel. When 2-mercaptoethanol was included in the electrophoresis sample buffer, the major band and the minor bands disappeared with the concomitant appearance of a sharp intensely labeled band with an apparent M_r of 49,000. These results may reflect either a decrease in the immunoreactivity of reduced PMR (M_r 215,000) or, alternatively, suggest that PMR as it exists in hamster liver may be an aggregate of a low molecular weight component(s).

An aliquot of hamster liver homogenate was also subjected to 2D-PAGE under reducing conditions and immunoblotted (Fig. 2*B*). Virtually all of the immunoreactive spots migrated with a M_r <215,000. The profile of immunoreactive spots

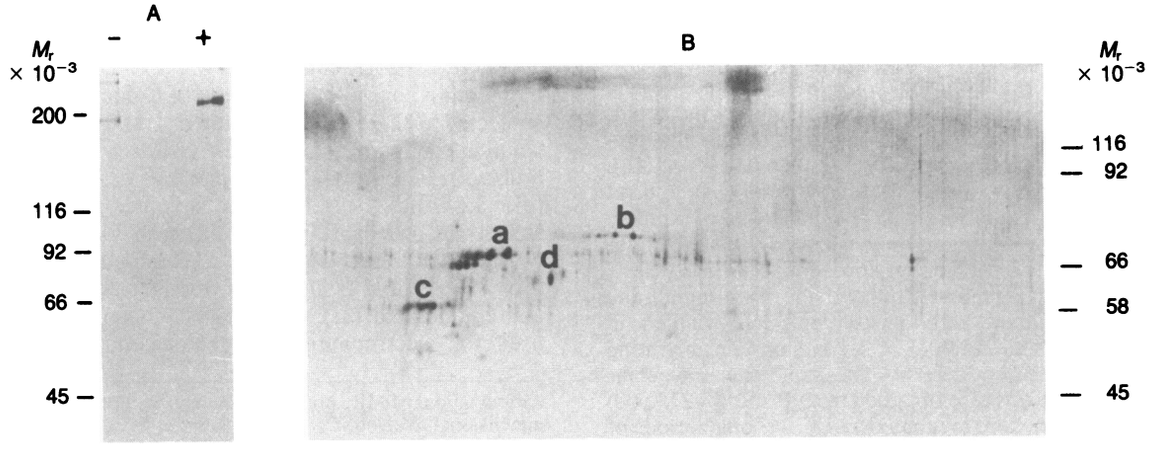

FIG. 1. Affinity-purified Syrian-hamster PMR [0.5 μg (*A*) and 2 μg (*B*)] were subjected to 1D-PAGE (*A*) in the presence (+) and absence (−) of 2-mercaptoethanol and 2D-PAGE (*B*). Proteins were visualized by silver staining (24). The position of each of the indicated molecular weight markers was determined using commercially available protein reference standards with the following M_r values: myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 92,000; bovine serum albumin, 66,000; and ovalbumin, 45,000.

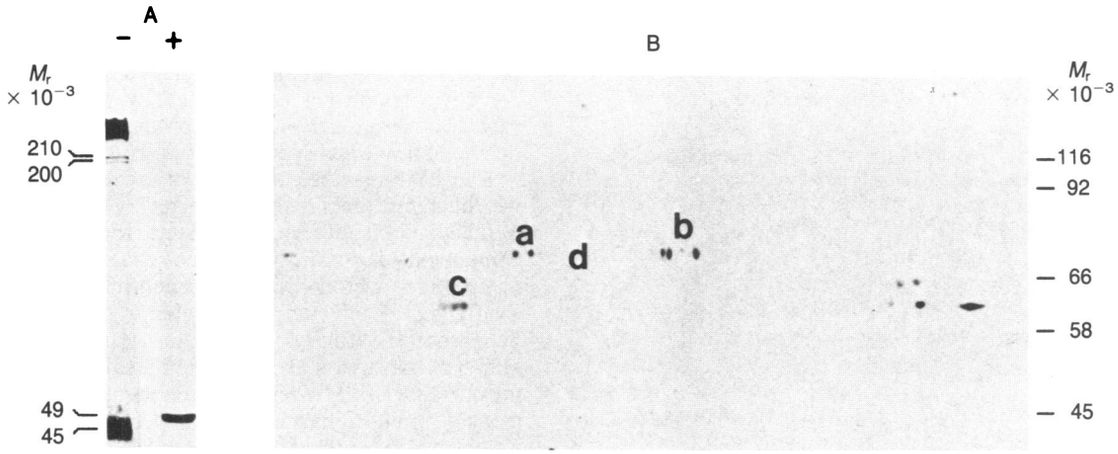

FIG. 2. Demonstration of PMR and immunochemically-related compounds in hamster liver by immunoblotting. Aliquots of liver homogenate (0.3 mg of protein) were subjected to 1D-PAGE (*A*) in the presence (+) or absence (−) of 2-mercaptoethanol or to 2D-PAGE (*B*) and immunoblotted. Antigen bands were visualized.

obtained closely resembled the profile obtained with affinity-purified hamster PMR (see Fig. 1*B*, areas a–d). Two additional spots with an approximate M_r of 62,000 were observed on the cathodic (right) side of the immunoblot. These spots were not observed in the corresponding region of the gel shown in Fig. 1*B*. The compounds may be related to components that are formed on the dissociation of hamster PMR on 2D-PAGE—i.e., may have been further metabolized resulting in altered pI values and/or possibly loss of ability to bind to the phosphomannan-Sepharose-4B affinity column. The weakly immunoreactive compound marked d corresponds in migration to protein d shown in Fig. 1*B*. No immunopositive spots were observed on 2D-PAGE gels treated with preimmune serum. Therefore, the bands observed in Fig. 2 (*A* and *B*) cannot be attributed to nonspecific antibody binding or to the presence of impurities in the antigen preparations used to immunize the rabbits. Furthermore, the presence of contaminating antibodies in the antiserum is unlikely since the PMR used to generate antibodies was isolated by affinity chromatography and purified additionally by electroelution of the 215,000 form of PMR from 1D-PAGE. That the multiple spots may arise from the action of proteinase(s) seems unlikely since prolonged incubation of hamster liver homogenates at 37°C did not result in the degradation of PMR—i.e., the appearance of low molecular weight components on immunoblots. That a major band (M_r 49,000) was observed on one-dimensional gels under reducing conditions but only a minor spot with a similar M_r was observed on two-dimensional gels is attributed to differences in sample preparation and running conditions between one- and two-dimensional gels.

Distribution of a Low Molecular Weight Immunoreactive Compound(s) in Hamster Liver Homogenates That Cross-React with Anti-PMR Antiserum. Homogenates of hamster liver were centrifugated at 130,000 × *g* at 4°C for 60 min. The resulting particulate and soluble fractions were subjected to 1D-PAGE under nonreducing conditions and immunoblotted (Fig. 3). A single major band(s) (M_r 43,000–49,000) was detected in the lanes containing whole homogenate and 130,000 × *g* supernatant. No band was detected in the lane containing solubilized particulate fraction. Small amounts of bands that migrated in the region of intact PMR were detected in both the supernatant and pellet fractions. The bands included a 205,000 component (PMR) present in the crude homogenate and the particulate fraction and a higher molecular weight compound observed in the supernatant.

Presence and Properties of Immunoreactive Low Molecular Weight Compound(s) in Acetone Powder Preparations and Hamster Serum and Plasma. When acetone powder preparations of hamster liver were extracted with Triton X-100 (16), subjected to 1D-PAGE, and immunoblotted, a low molecular weight component(s) was detected (Figs. 2*A* and 4). When the Triton X-100 extract was applied to the phosphomannan-Sepharose-4B affinity column, and the column flow through fractions, wash fractions, and Man-6-*P* eluate fractions (pooled and concentrated separately) were subjected to 1D-PAGE under reducing and nonreducing conditions, no low molecular weight material(s) was detected. Addition of high concentrations (2.0 M) of NaCl failed to elute the low molecular weight material. Only PMR (M_r 215,000) was detected in the Man-6-*P* eluate fraction. It is tempting to speculate that the experimental conditions used for affinity chromatography may favor protein association resulting in the formation of PMR aggregates and perhaps even insoluble macroaggregates.

Hamster serum was also subjected to 1D-PAGE, and the gels were immunoblotted (Fig. 4). A single component (M_r 185,000) was observed under nonreducing conditions; under

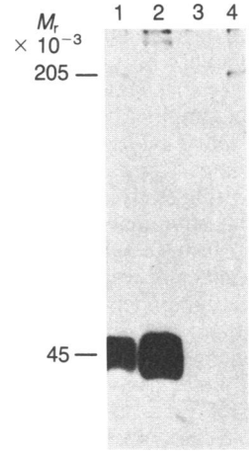

FIG. 3. Association of low molecular weight components that cross-react with antiserum generated against PMR (M_r 215,000). Hamster liver homogenate (150 μl) was centrifuged at 4°C in a Beckman airfuge for 30 min at 130,000 × *g*. The pellet was suspended in 0.05 M Tris-buffered saline, pH 7.4. Lanes: 1, 2 μl (0.3 mg of protein) of the hamster liver homogenate; 2, 2 μl of the 130,000 × *g* supernatant; 3, 2 μl of the 130,000 × *g* pellet; and 4, 4 μl of the 130,000 × *g* pellet. Following 1D-PAGE under nonreducing conditions, the proteins were subjected to immunoblotting with antiserum against PMR.

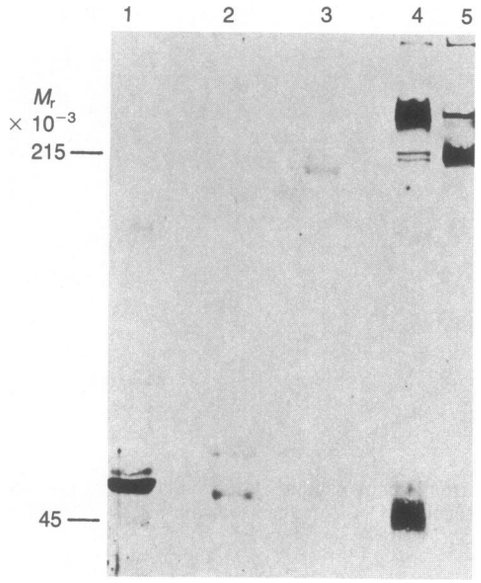

FIG. 4. Demonstration of PMR and immunochemically-related compounds in hamster serum by immunoblotting. Aliquots of purified PMR (1 μg, lane 5), liver homogenate (0.3 mg, lanes 1 and 4) and serum (2 μl, lanes 2 and 3) were subjected to 1D-PAGE in the presence (lanes 1 and 2) and absence (lanes 3–5) of 2-mercaptoethanol and immunoblotted with antiserum against PMR.

reducing conditions the major compound detected had a M_r of 49,000.

These results suggest that hamster PMR, as other receptors (29, 30), may exist *in vivo* in aggregates of varying molecular size and that under appropriate physiological conditions may dissociate giving rise to low molecular weight components.

DISCUSSION

The intracellular transport of newly synthesized lysosomal enzymes and the receptor-mediated endocytosis of extracellular lysosomal enzymes is thought to be mediated through the binding of Man-6-*P* of the oligosaccharide chains of these acid hydrolases. While alternative pathways for the "targeting" of lysosomal enzymes may exist (9), it is well documented that PMR plays a major role in the intracellular translocation and delivery of lysosomal enzymes (1–4).

Sahagian *et al.* (5) reported that bovine liver PMR, purified by affinity chromatography, has a M_r of 215,000. PMR preparations with similar molecular weights and properties have now been isolated from a variety of tissues (3). The results presented in this study and those of Mitchell *et al.* (18), Alvares and Balasubramanian (17), Sahagian *et al.* (5), and Creek and Sly (19) suggest that PMR is either comprised of aggregated low molecular weight components or, alternatively, low molecular components are tightly associated with PMR.

Although Syrian hamster PMR migrated in 1D-PAGE as a single band of high molecular weight, 215,000 (Fig. 1*A*), in 2D-PAGE (Fig. 1*B*) several spots with M_r values 45,000–90,000 were detected. These results are in contrast to those obtained by Steiner and Rome (6) with Swarm rat chondrosarcoma PMR. These investigators found only two isoelectric species each with an apparent M_r of 215,000. Mitchell *et al.* (18) demonstrated that (*i*) long term incubation at 4°C of bovine liver PMR in the presence of Zwittergent and in the absence of Man-6-*P* or (*ii*) lowering the pH and incubating under reducing conditions resulted in the formation of a spectrum of low molecular weight compounds. In the present studies, attempts to dissociate hamster or liver PMR into low molecular weight components by other than 2D-PAGE were unsuccessful. The above observations suggest that the avidity of the association of the components comprising PMR may vary from species to species. The situation could yet be further complicated by the observation that rabbit antiserum generated against hamster PMR exhibits a degree of tissue and species specificity (unpublished results).

That PMR may be comprised of associated low molecular weight components is also suggested by other investigations. Mitchell *et al.* (18) showed that a low molecular weight component (M_r ≈57,000) was coextracted with PMR from bovine liver acetone powder preparations by the detergent Zwittergent. Each compound bound to the phosphomannan-Sepharose-4B affinity matrix and was eluted with 5 mM Man-6-*P*. Alvares and Balasubramanian (17) demonstrated the presence of PMR-related components in homogenates of monkey brain. Sahagian *et al.* (5) observed low molecular weight components (related to PMR) in partially purified preparations of bovine liver, CHO cells, and rat hepatocytes. Distler and Jourdian (31) have purified a protein (M_r 42,000) from bovine testes by affinity chromatography that exhibits a binding specificity similar to that reported for bovine liver PMR.

In the present studies, low molecular weight components associated with, or comprising, PMR have been identified in intact hamster liver under reducing and nonreducing conditions as revealed by immunoblotting after 1D- and 2D-PAGE. When aliquots of hamster liver homogenates were subjected to 1D-PAGE under nonreducing conditions and immunoblotted, material migrating in the region of M_r 215,000 was demonstrable. Under reducing conditions no high molecular weight material was observed (Fig. 2*A*). Failure to visualize the high molecular weight material probably results from the observed diminished reactivity of the antibody with reduced PMR. The similarity of the migration profile of the labeled proteins on an immunoblot of a two-dimensional gel of crude hamster liver homogenate (Fig. 2*B*) to that of the purified PMR preparation (Fig. 1*B*) electrophoresed in the same manner suggests that the low molecular weight components recognized by the antibody are related to PMR. That the antibody is specific for PMR seems assured since after affinity chromatography the purified PMR was further purified by 1D-PAGE and the band corresponding to a M_r 215,000 was electroeluted and used to generate antibodies against PMR.

In experiments designed to show biosynthesis of PMR with pulse and chase studies, Creek and Sly observed synthesis of bands with M_r ≈45,000 and 94,000 after short pulse times (19). However, at longer pulse times, only synthesis of the 215,000 form of PMR was observed. The authors suggested that the low molecular weight bands were coprecipitated contaminants or degradative products derived from PMR. A possible alternative interpretation of these findings is that the low molecular weight materials represented components of PMR such as those demonstrated in the present studies. In support of the latter interpretation, Sahagian (4) found that CHO cells grown in the presence of NH_4Cl, a procedure that results in the accumulation of lysosomal enzymes in culture medium, accumulated low molecular weight proteins which cross-reacted with antibody to the PMR. The alteration of the pH of intracellular organelles by addition of NH_4Cl may interfere with the normal assembly of PMR. The apparent absence of low molecular weight bands (such as those described in the present studies) in pulse and chase experiments designed to study the biosynthesis of PMR (19, 32, 33) suggests that the low molecular weight bands do not arise from the action of proteinases on PMR of M_r 215,000.

Hamster liver homogenates and serum each contained an immunoreactive compound(s) of M_r 43,000–49,000 (Figs. 3 and 4). The 185,000 component found on immunoblots of hamster serum under nonreducing conditions dissociated

into a 46,000 band upon reduction (Fig. 4). These results support the concept that PMR is an aggregate comprised of low molecular weight components. It is tempting to speculate that this compound(s) may be the same or similar to one of the low molecular weight immunoreactive proteins found by Sahagian and co-workers (4, 5). Aggregation of such low molecular components could occur by an as yet unrecognized posttranslational modification and could account for the lag in the appearance of PMR (M_r 215,000) observed in pulse and chase experiments of Sahagian and Neufeld (33).

The above observations suggest that the low molecular weight components described in the present study, and those observed by others, are structurally and functionally related to PMR (M_r 215,000) and that PMR (M_r 215,000) is an aggregate as has been established for other receptors (29, 30).

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