Isolation and Characterization of Membranes from Bovine Liver Which Are Highly Enriched in Mannose 6-Phosphate Receptors

Donald J. Messner,* Gareth Griffiths,‡ and Stuart Kornfeld*

* Departments of Biological Chemistry and Internal Medicine, Division of Hematology, Washington University School of Medicine, St. Louis, Missouri 63110; and ‡Cell Biology Laboratory, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany

Abstract. We have developed a method for the isolation of the subcellular organelles from bovine liver which are enriched in the cation-independent mannose 6-phosphate receptor (CI-MPR) and the cation-dependent mannose 6-phosphate receptor (CD-MPR). The purification scheme consists of sedimentation of a postnuclear supernatant fraction on a sucrose gradient followed by immunoisolation using specific anti-peptide antibodies conjugated to magnetic polystyrene beads. Antibodies that recognize the cytoplasmic domain of either the CI-MPR or the CD-MPR routinely give membrane preparations that are ~50-fold enriched in each of the respective receptors, as determined by quantitative Western blotting. The immunoisolated membranes are also enriched in the other MPR, as well as in the asialoglycoprotein receptor. They contain significantly lower levels of enzyme activities representative of the plasma membrane (5′ nucleotidase) or the Golgi complex (galactosyltransferase and sialyltransferase). There is little or no enrichment for either the lysosomal enzymes β-hexosaminidase and tartrate-resistant acid phosphatase, or the mitochondrial enzyme succinate-tetrazolium reductase. These data, together with electron microscopy of the immunoisolated material, suggest that the bulk of MPR-containing membranes we have isolated from bovine liver correspond to endosomes. Analysis by SDS-PAGE indicates that several proteins, including two with apparent molecular weights of 170 K and 400 K, are significantly enriched in the purified fractions and may represent potential markers for MPR-containing endosomes.

MANNOSE 6-phosphate receptors (MPRs)[1] have a key role in the targeting of acid hydrolases to lysosomes in a variety of cell types (reviewed in references 37, 61). These receptors bind lysosomal enzymes bearing phosphomannosyl residues in the Golgi or on the cell surface, and then release these ligands when they encounter the low pH environment characteristic of newly forming lysosomes. Two distinct receptors have been identified (32, 54). The cation-independent MPR (CI-MPR) and the cation-dependent MPR (CD-MPR) are both integral membrane glycoproteins that appear to span the membrane bilayer a single time (9, 39, 53, 55). The lumenal (extracytoplasmic) domains form the bulk of the protein mass of both receptors, and are responsible for binding ligands. The CI-MPR and the CD-MPR have distinct amino acid sequences (9, 39, 45, 48), but regions of similarity suggest the two are homologous. The CI-MPR has a protein molecular weight of ~270,000 and binds ligands in a cation-independent manner (39, 45, 58). The CD-MPR is thought to be a functional homo-dimer, with each subunit having a protein molecular weight of ~30,000 (9, 48, 55). Its affinity for lysosomal enzymes is increased in the presence of divalent cations (59).

Much of what is postulated regarding the Man-6-dependent pathway has emerged from studying the subcellular distribution and routing of the Man-6-P receptors themselves, particularly the CI-MPR. It was originally postulated to be an endocytic receptor from studies on the cell surface binding and uptake of lysosomal enzymes (30). It has been localized in a variety of cell types to the Golgi stacks, the *trans*-Golgi network (TGN; also called *trans*-Golgi reticulum, *trans*-tubular network, or GERL), the plasma membrane, clathrin coated vesicles, and endosomes (6, 7, 8, 13, 19, 26, 53, 65), with the bulk of the receptor found in endosomes at steady-state. The receptor cycles through these compartments; surface labeling experiments have shown that all of the CI-MPR or CD-MPR are in single pools (12, 52, 56, 62). Neither MPR is present at a significant level in mature (dense) lysosomes, and so receptor-ligand dissociation is thought to occur in an earlier, prelysosomal, acidic compartment (7, 21, 55). Griffiths et al. (26) have shown that most of the CI-MPR in normal rat kidney (NRK) cells is localized in a late endosomal structure that contains lysosomal proteins and receives ligands internalized at the cell surface.

1. *Abbreviations used in this paper*: ASGP R, asialoglycoprotein receptor; CD, cation-dependent; CI, cation-independent; Man-6-P, mannose 6-phosphate; MPR, mannose 6-phosphate receptor; NRK, normal rat kidney; PNS, postnuclear supernatant; TGN, *trans*-Golgi network.

This specialized endosome may also receive newly synthesized lysosomal enzymes from the Golgi, and would therefore represent the junction point of the biosynthetic and endocytic pathways.

The distribution of the CD-MPR has been less well-studied than that of the CI-MPR. Preliminary immunolocalization experiments suggest that the CD-MPR, like the CI-MPR, is found in the TGN, the plasma membrane, and various endosomal structures (Griffiths, G., unpublished observations). It is thought to be important for sorting along the biosynthetic pathway, since cell lines which lack detectable levels of the CI-MPR but have the CD-MPR contain nearly normal levels of many lysosomal enzymes (15, 31). Furthermore, treatment of cells with antibodies which block the ligand binding site of the CD-MPR results in increased secretion of newly synthesized lysosomal enzymes (56). The CI-MPR has a higher affinity than the CD-MPR for most Man-6-P–containing ligands that have been examined to date (58, 59); there are no examples of lysosomal enzymes which will selectively bind to the CD-MPR. These points suggest a functional redundancy for the CI-MPR and the CD-MPR. However, the CD-MPR has been reported to not function in the endocytosis of lysosomal enzymes (56), even though it cycles between intracellular membranes and the cell surface (12, 56, 62). Perhaps the two receptors are enriched in functionally (and physically) distinct populations of endosomal structures. It is also possible that the two receptors use separate prelysosomal compartments to sort lysosomal enzymes to different classes of lysosomes.

Relatively little is known about the basic mechanisms governing receptor movement; an important first step is to define the biochemical characteristics of the organelles involved. With this in mind, we have developed procedures to purify membranes from bovine liver which are enriched in either the CI-MPR or the CD-MPR. The basic strategy was to perform immunoisolation using antibodies which recognize unique amino acid sequences of the cytosolic domains of each receptor. We have characterized the immunoisolated membrane preparations in an effort to: (*a*) establish whether the CI-MPR and the CD-MPR are present in the same, or different, populations of membranes, (*b*) determine the most likely cellular origin of these membranes, and (*c*) identify other proteins which might be enriched in these membranes and which could play a role in receptor movement and the targeting of lysosomal enzymes.

Materials and Methods

Materials

Peptides corresponding to various regions of the MPRs were synthesized, purified, and coupled to carrier proteins by Joe Bullock of Monsanto (St. Louis, MO). Dynabeads were obtained from Dynal, Inc. (Great Neck, NY). Na^{125}I and UDP-[^{3}H]galactose were from Amersham Corp. (Arlington Heights, IL). CMP-[^{3}H]sialic acid was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Iodogen was from Pierce Chemical Co. (Rockford, IL). Molecular weight standards for SDS-PAGE and the Bradford protein reagent were from Bio-Rad Laboratories (Cambridge, MA); other PAGE reagents came from ICN Biochemicals (Irvine, CA). Affinity-purified goat anti-rabbit IgG and all protease inhibitors were from Sigma Chemical Co. (St. Louis, MO). Other reagents were as noted or from standard suppliers.

Liver Homogenization and Density Gradient Centrifugation

Fresh calf liver was obtained from a local slaughterhouse and kept on ice until use. A portion (15–20 g) was excised and rinsed thoroughly with several hundred milliliters of homogenization buffer (25 mM Pipes, pH 7.4, 100 mM potassium chloride, 250 mM sucrose, 10 mM EDTA) containing a 1:500 dilution of protease inhibitor cocktail (5 mg/ml benzamidine and 1 mg/ml each of pepstatin A, leupeptin, antipain, and chymostatin in 40% dimethyl sulfoxide, 60% ethanol). The tissue was minced on ice and homogenized in 3 vol of buffer with 10–12 strokes of a loose fitting glass/teflon homogenizer (Wheaton Instruments Div., Millville, NJ). Unbroken cells and nuclei were removed by centrifugation at 600 *g* for 5 min. 60–70% of the total protein was routinely recovered in the postnuclear supernatant (PNS), which served as the starting material for fractionation on sucrose density gradients or immunoisolation. Some experiments (Fig. 4, *B* and *C*, and three of the six experiments summarized for Fig. 5) were performed using a postnuclear supernatant (PNS) fraction that had been frozen and thawed one time. The sucrose gradients were prepared with a standard linear gradient maker using 15 ml each of 15 or 45% sucrose in homogenization buffer containing a 1:1,000 dilution of protease inhibitor cocktail. The gradients were overlayed with ~2 ml of PNS (60–80 mg protein) and then with 5 ml of homogenization buffer minus sucrose. The tubes were centrifuged in a rotor (model SW27; Beckman Instruments, Inc., Palo Alto, CA) at 25,000 rpm (110,000 g_{max}) for 14–16 h at 4°C. Fractions were collected from the bottom of the tubes and the sucrose concentration determined with a refractometer. The protein concentrations of the initial homogenate, the PNS, and the sucrose gradient fractions were estimated using the method of Bradford (3) with BSA as standard.

Analysis of Marker Enzymes

β-Hexosaminidase activity was measured in 50 mM sodium citrate, pH 4.3, 0.2% Triton X-100 using 1 mM 4-methylumbelliferyl-*N*-acetyl-β-D glucosamide (Sigma Chemical Co.) as substrate. The reaction mixtures were incubated at 37°C, quenched with 0.2 M sodium carbonate, and the relative fluorescence (excitation at 360 nm, emission at 415 nm) determined in an Aminco fluorometer (S. L. M. Instrument Co., Urbana, IL). The procedure for measuring galactosyltransferase activity was a slight modification of that described by Bretz and Staubli (4). The donor substrate was UDP-D-[6-^{3}H]galactose (supplemented with nonradioactive material to 0.4 mM) and the acceptor was *N*-acetylglucosamine (50 mM). 5′ nucleotidase activity was determined according to the method described by Morre (42) using 10 mM AMP in 50 mM Tris, pH 8.5, 10 mM magnesium chloride, 10 mM sodium/potassium tartrate, and 0.2% Triton X-100. The inorganic phosphate produced was measured by the method of Fiske and SubbaRow (as described in reference 38). Acid phosphatase activity was determined by dilution of the samples into 100 mM sodium acetate, pH 5.0, 2 mg/ml BSA, 0.2% Triton X-100, 1 mM EDTA, 10 mM *p*-nitrophenyl phosphate (Sigma Chemical Co.) and either 10 mM sodium chloride or 10 mM sodium/potassium tartrate (16). The samples were incubated at 37°C, the reaction quenched with 0.5 M glycine, pH 10.4, and the absorbance read at 405 nm. The difference in activity with and without tartrate was taken as tartrate-inhibitable acid phosphatase activity. Succinate-INT reductase activity was measured as described (46). CMP-NeuAc:glycoprotein sialyltransferase activity was also determined as described (5), using asialofetuin as acceptor (27). The incorporation of [^{3}H]sialic acid was nearly completely dependent on the exogenously added substrate.

PAGE, Immunoblotting, and Receptor Quantitation

The conditions for PAGE in SDS were as described by Maizel (40). All samples were denatured by boiling in SDS sample buffer containing 100 mM 2-mercaptoethanol for 5 min. Silver staining was performed according to Oakley (44). For immunoblotting, the 8% polyacrylamide gels were equilibrated in transfer buffer (150 mM glycine, 20 mM Tris, 20% methanol, pH 8.3) and transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) in a Transblot apparatus (Bio-Rad Laboratories) at 30–35 V for 8–14 h, similar to the method first described by Towbin (60). Control experiments established that under these conditions >80% of ^{125}I-labeled CI-MPR and CD-MPR added in trace amounts to 50 μg PNS protein were transferred to the blot (data not shown). Subsequent treatment of the nitrocellulose blot varied with the protein being examined. For the CI-MPR and the ASGP R, the blot was incubated for 2 h in blotto (5% [wt/vol] nonfat dry milk (Carna-

tion), 50 mM Tris, pH 7.8, 0.05% Triton X-100, 2 mM calcium chloride, and 0.01% antifoam [Sigma Chemical Co.]). This and all the following steps were performed on a rotary shaker at room temperature. The blots were rinsed with fresh blotto, and the appropriate antisera was applied. The anti-CI-MPR was diluted 1:200 in blotto and the anti-ASGP R was diluted 1:100 in blotto; each preparation was used for many blots and was stored at −20°C between uses. The antibodies were allowed to bind for 2 h, the blots were washed five to six times (5 min each) in blotto, and 1×10^7 cpm of ^{125}I-labeled goat anti-rabbit IgG (prepared as described below) was added in blotto. After a 1-h incubation, the blots were washed three times (10 min each) in blotto and four times (5 min each) in PBS (50 mM sodium phosphate, pH 7.5, 150 mM sodium chloride). The nitrocellulose was allowed to air dry, wrapped in cellophane, and put down for autoradiography with Kodak X-OMAT AR film.

Various protocols were used for the CD-MPR blots; all were similar to that described above. The best results were obtained by washing in 1% polyvinylpyrrolidone 40 (Sigma Chemical Co.) in PBS after the initial blocking of the nitrocellulose with blotto. The blots were then incubated in a preparation of affinity-purified anti-CD-MPR antibodies (see below) in this buffer for 4–12 h at room temperature. The washing steps were performed with PBS first with, and then without, 1% polyvinylpyrrolidone 40, and the ^{125}I-labeled goat anti-rabbit IgG was added in 1% polyvinylpyrrolidone 40 in PBS. The final washes consisted of once in 1% polyvinylpyrrolidone 40 in PBS, once in PBS, once in 0.025% Triton X-100 in PBS (all for 10 min each), and four times (5 min each) in PBS.

The blots were quantified by using the autoradiogram as a template to cut out the relevant bands, which were then counted in a gamma counter. To compensate for variations in the size (and, therefore, in the background) of the excised bands, a blank region of the blot was excised, counted, and weighed on an analytical balance to determine background counts per minute per microgram of nitrocellulose. This value was multiplied by the weight of each excised band to determine the nonspecific background signal for that band, which was then subtracted from the total counts per minute to give the specific signal. This procedure gave linear responses in control experiments with all three antibody preparations using up to 50 μg PNS protein per gel lane (see Fig. 1). In general, the specific signals were determined relative to an aliquot of the starting material; linearity was verified on each blot by including aliquots corresponding to 20 and 100% of the initial sample.

Generation and Purification of Antisera

Antisera against the native CI-MPR was prepared by multiple injections of a single rabbit with CI-MPR purified from bovine liver as described (32), except that the initial chromatography was performed on an Affi-Gel column conjugated with phosphopentamannose, not phosphomannan. The anti-CD-MPR was prepared using CD-MPR, also purified from bovine liver, that had been denatured with SDS and 2-mercaptoethanol. The anti-CD-MPR antibodies were purified by precipitation in 40% ammonium sulfate; the pellet was resuspended in 20 mM sodium phosphate, pH 7.0, 100 mM sodium chloride, 0.02% sodium azide, and dialyzed against this buffer (57). The antibodies were affinity purified on a column of denatured CD-MPR coupled to cyanogen bromide–activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to the supplier's instructions. Anti-CD-MPR antibodies were eluted with 0.2 M glycine, pH 2.5, and immediately neutralized with 1 M Tris base. Preparations of anti-ASGP R sera were generously provided by Dr. Alan Schwartz (Washington University) and Dr. Kurt Drickamer (Columbia University). All antisera and affinity-purified antibodies were stored at −20°C until use.

The synthetic peptides used to generate antisera were conjugated to keyhole limpet hemocyanin by one of two methods. Method A: CI-MPR peptides Nos. 1 and 2 were synthesized with an extra cysteine residue at the amino terminus. The carrier protein was alkylated with iodoacetamide and desalted on Sephadex G-25. 10 mg was combined with 0.28 mg *N*-succinimidyl-3(2-pyridyldithio)proprionate in 0.02 ml ethanol over 45 min, and desalted into 25 mM sodium phosphate, pH 7.5, 150 mM sodium chloride (0.025 M PBS). An equal weight amount of peptide was added and incubated for 20 h at 4°C. The mixture was dialyzed against 0.025 M PBS and stored at −20°C. Method B: The CD-MPR peptide (5 mg) was combined with 10 mg of carrier protein in 2 ml 0.025 M PBS, and 0.5 ml of 20 mM glutaraldehyde was added dropwise at 20°C. The mixture was incubated for 1 h, and then dialyzed and stored as above.

The anti-peptide sera were raised by multiple injections of the peptide/keyhole limpet hemocyanin conjugates in rabbits. The antigen (1 mg peptide/ml) was mixed in a 1.0:1.5 ratio with Freunds complete adjuvant (Gibco Laboratories, Grand Island, NY) to give a thick emulsion. This mixture (containing 200–300 μg peptide) was injected at weeks 0, 3, and 5. Depending on the response of each rabbit, subsequent injections (of 100–200 μg peptide) were made at weekly to monthly intervals. Blood was taken 7–10 d after each boost. Titers were estimated by immunoprecipitation of purified and ^{125}I-labeled CI-MPR or CD-MPR (see below). The anti-CI-MPR peptide No. 1 preparation (see Fig. 3) was derived from the serum of one of two rabbits which produced anti-CI-MPR antibodies; two additional rabbits did not respond. The anti-CI-MPR peptide No. 2 preparation came from the pooled sera of both rabbits that were immunized. The anti-CD-MPR peptide preparation came from pooled sera from two of the four rabbits which responded to that conjugate. A second CD-MPR peptide, corresponding to the carboxy-most region of the protein, failed to elicit antibodies capable of recognizing the mature protein in any of the four rabbits injected. All three anti-peptide sera were purified by ammonium sulfate precipitation and chromatography on MPR-Sepharose columns as described above.

Proteins were labeled with ^{125}I using the Iodogen procedure (14). 100 μg of protein in 50 mM Tris, pH 7.4, 100 mM sodium chloride was added to a tube containing 20 μg of Iodogen, 5 μl 1 M Tris, pH 8.0, and 0.5 mCi Na^{125}I (carrier free). The reaction was allowed to proceed for 10 min at room temperature, and the mixture was desalted on a 10 ml Sephadex G-25 column equilibrated in 1 mg/ml BSA, 50 mM Tris, pH 7.4, 100 mM sodium chloride, and 0.02% sodium azide. For the labeled antibodies, the protein peak was pooled and used directly. For the MPRs, 0.05% Triton X-100 was included in the columns, and the protein peak was dialyzed extensively against 50 mM Imidazole, pH 6.5, 100 mM sodium chloride, 5 mM β-glycerophosphate, 0.02% sodium azide containing 10 mM EDTA (for the ^{125}I-CI-MPR) or 10 mM manganese chloride (for the ^{125}I-CD-MPR). The labeled receptors were then isolated by chromatography on phosphomannan-Sepharose in the appropriate buffer and eluted with 5 mM Man 6-P as has been described (32).

Dynabead Preparation, Immunoisolation, and Recovery Calculations

Uncoated Dynabeads M450 (Dynal, Inc.) were activated with *p*-toluenesulphonylchloride and pyridine in acetone according to the directions supplied by the manufacturer. Approximately 300 mg of activated beads were then resuspended in water and an equal volume of 0.2 M sodium borate, pH 9.5, containing 5 mg of affinity-purified goat anti-rabbit IgG (Sigma Chemical Co.) was added. The reaction was allowed to proceed for 1 d at room temperature, after which the Dynabeads were isolated and washed in 100 mM sodium phosphate, pH 7.4, 100 mM sodium chloride. The unreacted sites were blocked by incubation in 1 M ethanolamine, pH 9.5, for 2 h, and the beads were washed several times with 50 mM Tris, pH 7.4, 100 mM sodium chloride, 1 mg/ml BSA, and 0.02% sodium azide, first with 0.1% Tween 20 and then without. The Dynabead anti-rabbit antibody preparation was stored in this buffer at 4°C until use.

Immediately before use, the Dynabeads were washed several times in wash buffer (25 mM Pipes, pH 7.4, 100 mM potassium chloride, 500 mM sucrose, 10 mM EDTA, 0.5% polyvinylpyrrolidone 40, and a 1:1,000 dilution of protease inhibitor cocktail). The beads were saturated with an excess of affinity-purified anti-peptide antibody (or nonimmune sera) in wash buffer by end over end rotation for 5–6 h at 4°C. The final Dynabead/anti-rabbit/anti-peptide antibody conjugates were washed several times with wash buffer and then used for immunoisolation. The relative amounts of protein and Dynabeads used varied from experiment to experiment and are noted in the figure legends. In general, PNS samples were diluted to 1–2 mg/ml in Dynabead wash buffer; fractions from the sucrose gradient were diluted with sucrose-free homogenization buffer to a final concentration of 0.5 M sucrose, and diluted further to 0.3–0.5 mg/ml protein with 25 mM Pipes, pH 7.4, 100 mM potassium chloride, 500 mM sucrose, 10 mM EDTA, and a 1:1,000 dilution of protease inhibitor cocktail. The experiment illustrated by Table II was slightly different; the sucrose gradient fractions had been stored at −20°C and thawed before immunoadsorption and the protein concentration was 1.0 mg/ml. Unless otherwise noted, immunoisolation was allowed to proceed with constant mixing overnight (12–14 h) at 4°C. The Dynabeads were isolated with a magnet and the supernatant was removed and saved for analysis. The Dynabeads were washed twice by gentle resuspension in wash buffer and end over end rotation at 4°C for 30–40 min.

The washed beads were generally resuspended in half the volume of the supernatant and analyzed in immunoblots or for enzyme activities in paral-

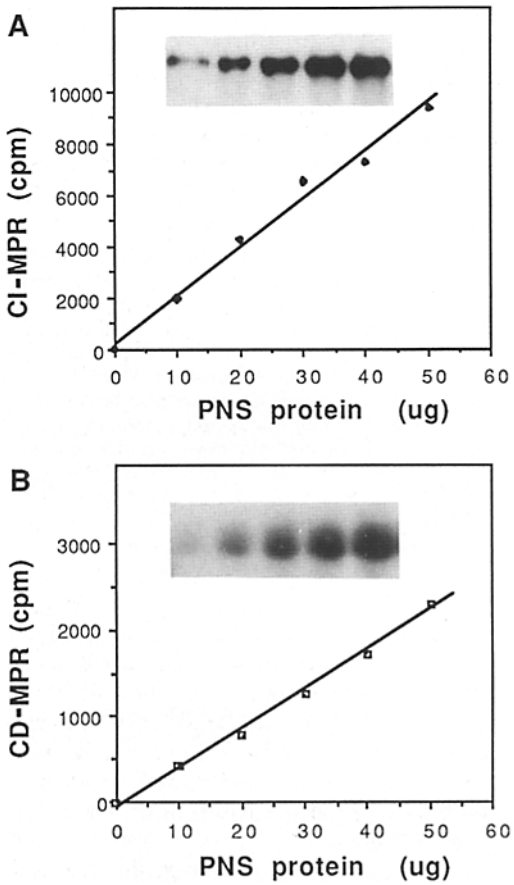


Figure 1. MPR quantitation in bovine liver PNS. The indicated amount of PNS protein was electrophoresed on 8% polyacrylamide gels, transferred to nitrocellulose, and subjected to immunoblotting with specific antibodies followed by ^{125}I-labeled second antibodies as described in Materials and Methods. The labeled bands were excised from the blot and the specific ^{125}I signal determined using a gamma counter. The best line was determined by least-squares analysis. The insets contain autoradiograms of the regions of the blots which were quantified in this fashion. (*A*) CI-MPR. (*B*) CD-MPR.

lel with the supernatants and aliquots of the initial fractions. The protein content was determined by elution of the beads with Triton X-100 and analysis of these extracts by the method of Peterson (47). The fold-purification data presented in Table I were calculated by dividing the yield of each activity through the two step procedure by the yield of protein. The yield of each component is defined as the percent of total on the gradient found in pool IV times the percent adsorbed onto the Dynabeads. Some of the experiments used to generate Table I are shown in Fig. 8. The yield data presented in Table II are for the immunoisolation step only. The input material was a sucrose gradient pool which contained the indicated percentages of each respective component as listed under the activity column of Table II. The percentages of protein, sialyltransferase, succinate-INT reductase, and acid phosphatase activities were determined on aliquots of the fractions used for immunoisolation; the remaining components were measured on a parallel gradient.

Electron Microscopy

Aliquots of PNS adsorbed to the Dynabeads were pelleted in a microfuge and overlayed with a solution of 8% paraformaldehyde, 0.2 M Pipes, pH 7.0, for 60 min. The fix solution was changed one time, and the samples were stored at 4°C or above. For Fig. 6 *A*, the fixed samples were rinsed with 1% osmium tetraoxide in 0.1 M sodium cacodylate, dehydrated in ethanol, and embedded in Epon (25). For the immunogold labeling (Fig. 6, *B* and *C*), the pellets were infused with 2.1 M sucrose and prepared for cryosectioning as has been described (24). The sections were cut with a "cryo" diamond knife (Diatome Co., Bienne, Switzerland), thawed, and treated with 5% newborn calf serum followed by 50 μg/ml protein A to block nonspecific antibody or protein A binding sites on the beads. The specific antibodies used were (*a*) anti-CI-MPR antisera (as described in reference 26), and (*b*) affinity-purified rabbit antibodies to the bovine CD-MPR (a generous gift of Bernard Hoflack, EMBL). These were used at a dilution of 1:5. Single and double labeling was done according to the method of Geuze et al. (17). The sections were contrasted and dried in methyl cellulose (24); in a few cases they were negatively stained in 1% ammonium molybdate in 2% methylcellulose (15 cp).

Results

Quantitation and Initial Fractionation of the MPR-containing Membranes of Bovine Liver

Bovine liver is an easily obtainable tissue that is an excellent source for the purification of both the CI-MPR and the CD-MPR (32). Since these two proteins differ significantly in both molecular weight and immunological properties (32), they can be individually quantified by immunoblotting. Fig. 1 shows the results of the blotting protocols we have developed as applied to a total PNS fraction from bovine liver. In each case, a single major band is identified with the apparent molecular weight of the corresponding MPR purified from bovine liver (not shown) and the signals obtained are linear over a suitable range of PNS protein (0–50 μg). These procedures can quantitatively distinguish between the CI-MPR and the CD-MPR in mixed preparations of the two, a requirement we were not able to meet with any of a variety of ligand binding procedures examined (e.g., see references 15, 58). Quantitative immunoblotting, therefore, has been our method of choice for documenting the purification of CI-MPR- and CD-MPR-containing membranes from bovine liver.

Fractionation of bovine liver PNS by sedimentation on a 15–45% linear sucrose gradient gave the results shown in Fig. 2. This procedure clearly separates the bulk of the CI-MPR and the CD-MPR (Fig. 2 *A*) from dense lysosomes and cytosolic protein (Fig. 2 *B*). It should be noted that a significant fraction of the β-hexosaminidase remained at the top of the gradient, indicative of leakage from or partial rupture of lysosomes.[2] Both MPRs comigrate to varying extents with markers for the Golgi complex (galactosyltransferase), endosomes (the ASGP R), and the plasma membrane (5′ nucleotidase) in this gradient system (Fig. 2 *C*). The two MPRs have distinct profiles; the CI-MPR is broadly distributed in fractions 4–8, while the CD-MPR is particularly enriched in fractions 7 and 8. When fractions 6–9 were pooled, the CI-MPR and the CD-MPR were purified approximately four- and sixfold, respectively, over the initial PNS fraction. Since this pool consisted of a variety of liver membranes with very similar physical properties, we reasoned that further purification would require a specific technique capable of separating the membrane vesicles and organelles on the basis of their MPR content, rather than size or density. To accomplish this, we took advantage of the fact that each of the MPRs contains a portion which is exposed to the cytoplasmic

2. The fractionation of rat liver PNS has been examined in similar experiments; <10% of the total β-hexosaminidase activity remained at the top of the gradient. It appears, therefore, that a significant fraction of the bovine liver lysosomes are less stable than those from the rat.

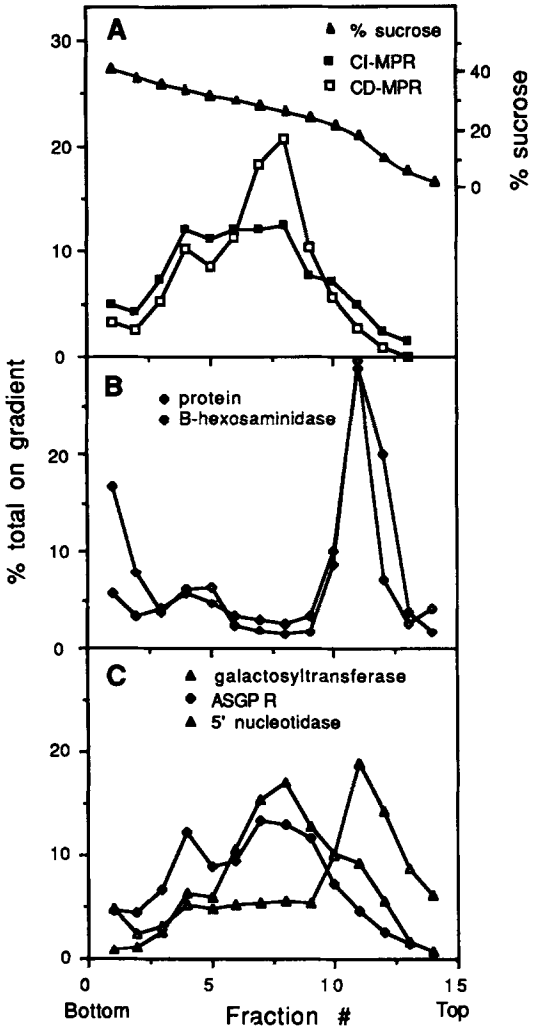


Figure 2. Fractionation of bovine liver PNS by sedimentation on sucrose gradients. An aliquot of PNS (60 mg protein) prepared from bovine liver was sedimented at 110,000 *g* for 14 h on a 15–45% linear sucrose gradient. The fractions were collected from the bottom and analyzed for the indicated constituent as described in Materials and Methods. The components examined and their recoveries on the gradient (expressed as the percent of each applied) are as follows: (*A*) CI-MPR (■), 71% recovered; CD-MPR (□), 101% recovered; sucrose (△) concentrations. (*B*) Total protein (♦), 100% recovered; β-hexosaminadase (◇), 81% recovered. (*C*) ASGP R (♦) 60% recovered; galactosyltransferase (▲), 90% recovered; 5′ nucleotidase (△), 55% recovered.

surface. This allowed the development of a specific immunoisolation procedure.

Generation of Antisera Specific for the Cytoplasmic Domains of the MPRs and Development of the Immunoisolation Procedure

The complete amino acid sequences of the bovine CI-MPR and CD-MPR have been deduced from their cDNA clones (9, 39). This information, together with a variety of biochemical experiments, indicates that both MPRs are oriented with the bulk of their mass on the lumenal side of intracellular membranes (and the cell exterior of the plasma membrane), where these regions participate in ligand binding (9, 53, 55). The orientation of intracellular membranes after homogenization is such that the much smaller cytoplasmic domains of the MPRs would be expected to be exposed in a vesicle or organelle preparation. To obtain antibody preparations with the highest possible titer towards these structures, a series of synthetic peptides corresponding to several regions of the cytoplasmic domains of the CI-MPR and CD-MPR were used as antigens (as described in Materials and Methods). Three of the peptides resulted in antisera that proved to be capable of specifically recognizing the corresponding mature protein. Fig. 3 *A* illustrates the approximate location of these peptides in the CI-MPR and the CD-MPR. Immunoprecipitation experiments using purified and ^{125}I-labeled MPRs established that each antibody recognizes only the "correct" MPR, and does not cross react with the other (Fig. 3 *B*). The two different anti-CI-MPR peptide antibodies gave very similar results in all of our experiments; in general we present data from only one of these for simplicity.

The immunoisolation matrix chosen for these experiments is a commercially available polystyrene bead embedded with a ferric oxide material (Dynabeads) which can be rapidly removed from a suspension of membranes with a standard bar magnet. Goat anti-rabbit antibodies were chemically conjugated to the Dynabeads and saturated with affinity-purified anti-MPR peptide antibodies as described in Materials and Methods. Initial experiments to optimize the conditions used for immunoisolation were performed using the PNS fraction prepared from bovine liver; some of these are shown in Fig. 4. In general, the extent of immunoadsorption was evaluated by quantitative Western blotting of the Dynabead-bound and supernatant fractions. Near maximal immunoadsorption of the MPR-containing membranes was achieved by overnight incubation with ~5 mg of antibody-saturated Dynabeads per mg of PNS protein (Fig. 4, *A* and *B*). Under these conditions, ~40% of the total CI-MPR could be bound to the anti-CI-MPR peptide beads, while nearly 20% of the CD-MPR bound to the anti-CI-MPR peptide beads. Nonspecific binding of membranes to the beads was very low (only a few percent) as determined by using Dynabeads which had been prepared with nonimmune sera (Fig. 4). Specific immunoadsorption could be blocked in each case by including an excess of the relevant peptide in the incubation, while a nonrelevant peptide had no significant effect (Fig. 4 *C*).

A Population of Liver Membranes Contains Both the CI-MPR and the CD-MPR

Analysis of the various immunoadsorbed preparations revealed that the CD-MPR was enriched in membranes isolated using the anti-CI-MPR peptide antibodies, and the CI-MPR is enriched in membranes isolated on the basis of their CD-MPR content (Fig. 5). This was not observed when the membranes were solubilized with 1% Triton X-100 (not shown). When the total PNS fraction was immunoadsorbed, there was roughly twice the percent of total CI-MPR compared to the percent of total CD-MPR on the Dynabeads. This ratio is approximately the same in membranes isolated with antibodies specific for either receptor (Fig. 5, *inset*). Furthermore, sequential or concurrent immunoadsorption with both the anti-CI-MPR beads and the anti-CD-MPR beads did not result in a yield significantly greater than that obtained with a comparable level of one or the other alone

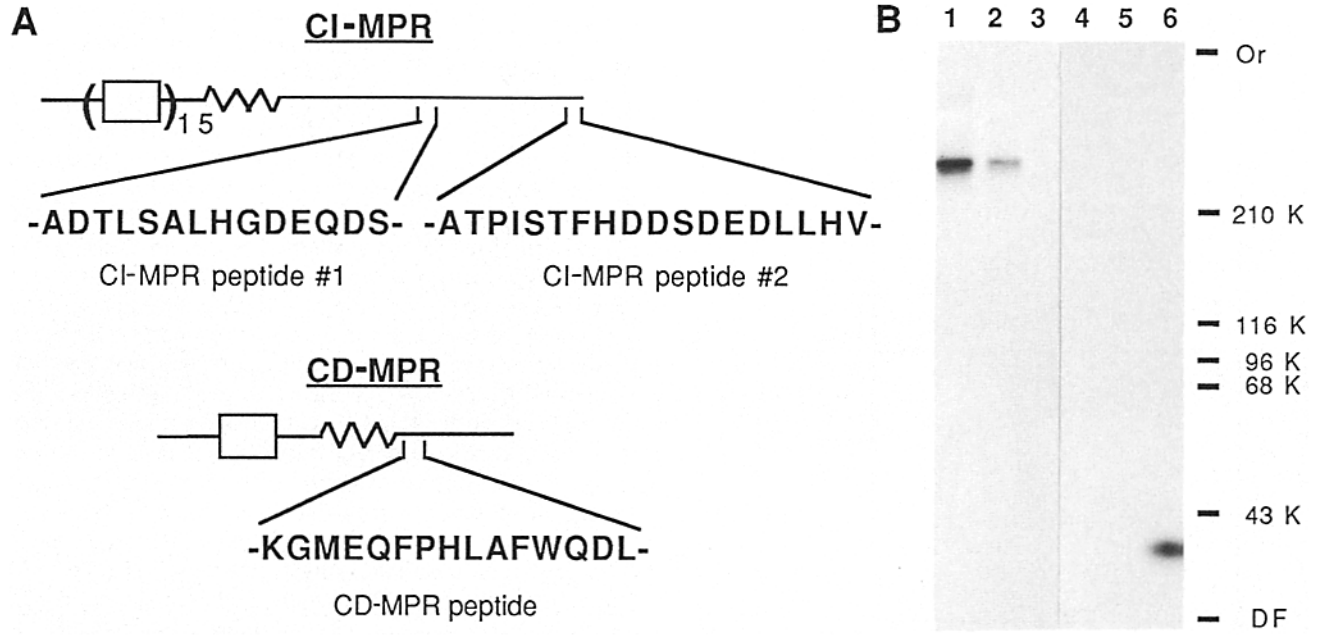


Figure 3. Generation of anti-peptide sera that recognize the mature CI-MPR and CD-MPR. (*A*) The two MPRs are diagrammed schematically with the homologous repeating units (represented by rectangles), the putative transmembrane regions (as jagged lines), and the carboxy-terminal cytoplasmic tails containing the peptides used in this study. The amino acid sequences of the peptides are given. The peptides have been designated as CI-MPR peptide Nos. 1 and 2, which correspond to residues 2408-2421 and 2482-2499, respectively, of the 2,499-amino acid sequence deduced from the cDNA clones obtained for the bovine CI-MPR (39). The CD-MPR peptide corresponds to residues 198–212 of the 257-amino acid CD-MPR sequence, also as deduced from cDNA clones (9). (*B*) Immunoprecipitation of ^{125}I-CI-MPR and ^{125}I-CD-MPR by the anti-peptide sera. Equivalent aliquots of ^{125}I-CI-MPR or ^{125}I-CD-MPR were immunoprecipitated with saturating levels of each of the peptide sera, followed by protein A–Sepharose as described in Materials and Methods. The immunoprecipitates were boiled in SDS sample buffer and subjected to SDS-PAGE on a gradient gel of 4–12% acrylamide, followed by autoradiography at −70°C for 19 h (lanes *1–3*) or 6 h (lanes *4–6*). The antisera and percent of total ^{125}I-MPR immunoprecipitated in each case are as follows: *lane 1*, anti-CI-MPR peptide No. 1, 20% of total ^{125}I-CI-MPR; *lane 2*, anti-CI-MPR peptide No. 2, 9% of total ^{125}I-CI-MPR; *lane 3*, anti-CD-MPR peptide, <1% of total ^{125}I-CI-MPR; *lane 4*, anti-CI-MPR peptide No. 1, <1% of total ^{125}I-CD-MPR; *lane 5*, anti-CI-MPR peptide No. 2, <1% of total ^{125}I-CD-MPR; *lane 6*, anti-CD-MPR peptide, 39% of total ^{125}I-CD-MPR. The maximal level of ^{125}I-CI-MPR capable of being immunoprecipitated by the anti-CI-MPR peptide sera (particularly anti-CI-MPR peptide No. 2) is somewhat low, and is variable from preparation to preparation of CI-MPR. This value is very reproducible for a given batch of ^{125}I-CI-MPR. In addition, we have observed that the cytoplasmic domain of the Triton-solubilized CI-MPR is highly susceptible to proteolysis by added trypsin. It is likely, therefore, that a certain amount of degradation of the cytoplasmic domains occur during homogenization and purification, despite the extensive use of protease inhibitors, and that this prevents quantitative immunoprecipitation of the ^{125}I-MPRs.

(data not shown). These findings indicate that the bulk of the membranes isolated by the anti-CI-MPR beads are also isolated by the anti-CD-MPR beads. Since the efficiency of immunoisolation has been shown to be highly dependent on the surface density of antigen (28), we conclude that the purified preparations represent those membranes with the highest density of antibody-accessible CI-MPR and CD-MPR in the liver. Clearly both receptors are enriched in the same membranes.

Analysis of the immunoadsorbed PNS fractions by electron microscopy (Fig. 6 *A*) revealed that the purified membranes consisted of a heterogeneous population of tubules and vesicles that were not present on the control (nonimmune) beads (not shown). The membranes were indistinguishable regardless of which MPR antibody was used in the immunoisolation. Readily identifiable organelles, such as mitochondria, plasma membrane sheets, or Golgi stacks were absent or infrequently detected. Immunogold labeling of thin frozen sections prepared from the purified membranes with specific anti-receptor antisera and protein A-gold confirmed the presence of membranous structures which contain the CI-MPR and the CD-MPR (Fig. 6, *B* and *C*). The specificity of the immunolabeling was verified by the much lower levels of gold particles when the sections were (*a*) treated with protein A-gold in the absence of the specific antibodies, or (*b*) treated with either affinity-purified antibodies against the vesicular stomatitis virus "G" protein or an antiserum against the spike-protein complex of Semliki forest virus, followed by protein A-gold. When these controls were quantified (22), there was an average of 0.92 ± 0.45 gold particles/μm^2 membrane (mean ± SEM, n = 23 micrographs) for membrane preparations isolated on the basis of either the CI-MPR or the CD-MPR content. This compares with the signals obtained using the anti-CI-MPR antibody for immunolabeling of 8.43 ± 1.47 (CI-MPR-enriched membranes, n = 34) and 8.63 ± 1.44 (CD-MPR-enriched membranes, n = 41). The corresponding signals using the anti-CD-MPR antibody were 3.39 ± 0.95 (CI-MPR-enriched membranes, n = 16) and 2.84 ± 0.60 (CD-MPR-enriched membranes, n = 20). These data supply additional evidence to support our conclusion that the CI-MPR shares the same membrane as the CD-MPR.

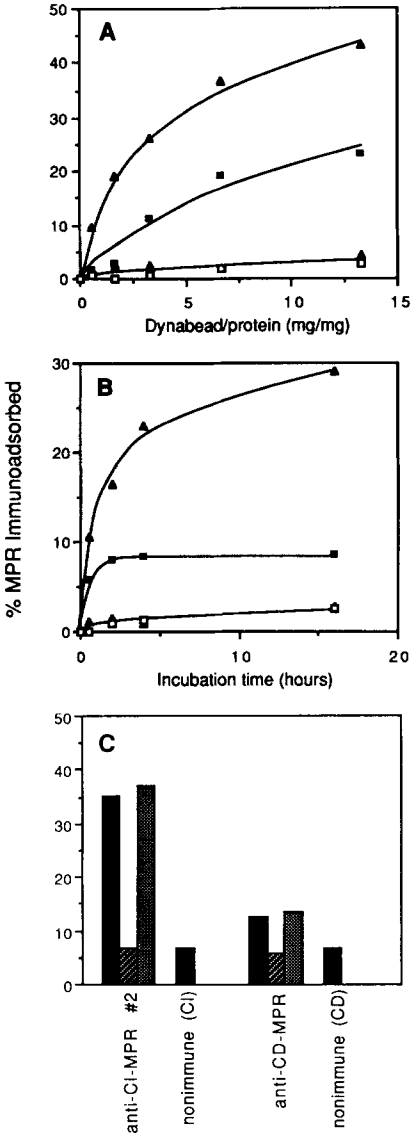


Figure 4. Immunoisolation of MPR-enriched membranes using Dynabeads. (*A*) Fraction of the total MPR absorbed by saturating Dynabeads. Dynabead conjugates were prepared with affinity-purified anti-CI-MPR peptide No. 2. (▲), anti-CD-MPR peptide (■), or nonimmune sera (△, □), and incubated with bovine liver PNS that had been diluted to 1 mg protein/ml. The indicated amount of Dynabeads were incubated overnight with 1 mg PNS protein, isolated with a magnet, washed, and prepared for SDS-PAGE as described in Materials and Methods. After electrophoresis, the samples were transferred to nitrocellulose and subjected to immunoblotting with anti-CI-MPR (*triangles*) or anti-CD-MPR (*squares*), followed by ^{125}I-labeled second antibody. The relative amount of MPR in each sample was quantified in a gamma counter and is expressed as a percentage of the starting material. (*B*) Time course of immunoisolation. Two milligrams of each Dynabead preparation were incubated with 1 mg PNS protein for the indicated time and analyzed as described above. The symbols are identical to those used in *A*. (*C*) Block of immunoisolation by the peptides used to generate the anti-MPR sera. A 2-mg aliquot of the indicated Dynabead preparation was preincubated for 30 min alone (■), or with 100 μg of either the peptide used to generate the antisera (▨) or a nonrelevant peptide (NH_2-CFYLFEMDSSLAC-COOH) (▩) in Dynabead wash containing 0.25 M sucrose. One milligram of PNS protein was added, the mixtures were incubated overnight, and then analyzed as described above to determine the percentage of total CI-MPR immunoadsorbed on the anti-CI-MPR peptide beads and the percentage of total CD-MPR immunoadsorbed to the anti-CD-MPR peptide beads. The nonimmune beads were analyzed for both MPRs as indicated.

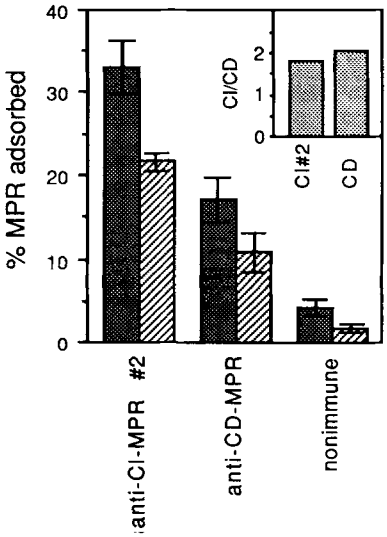
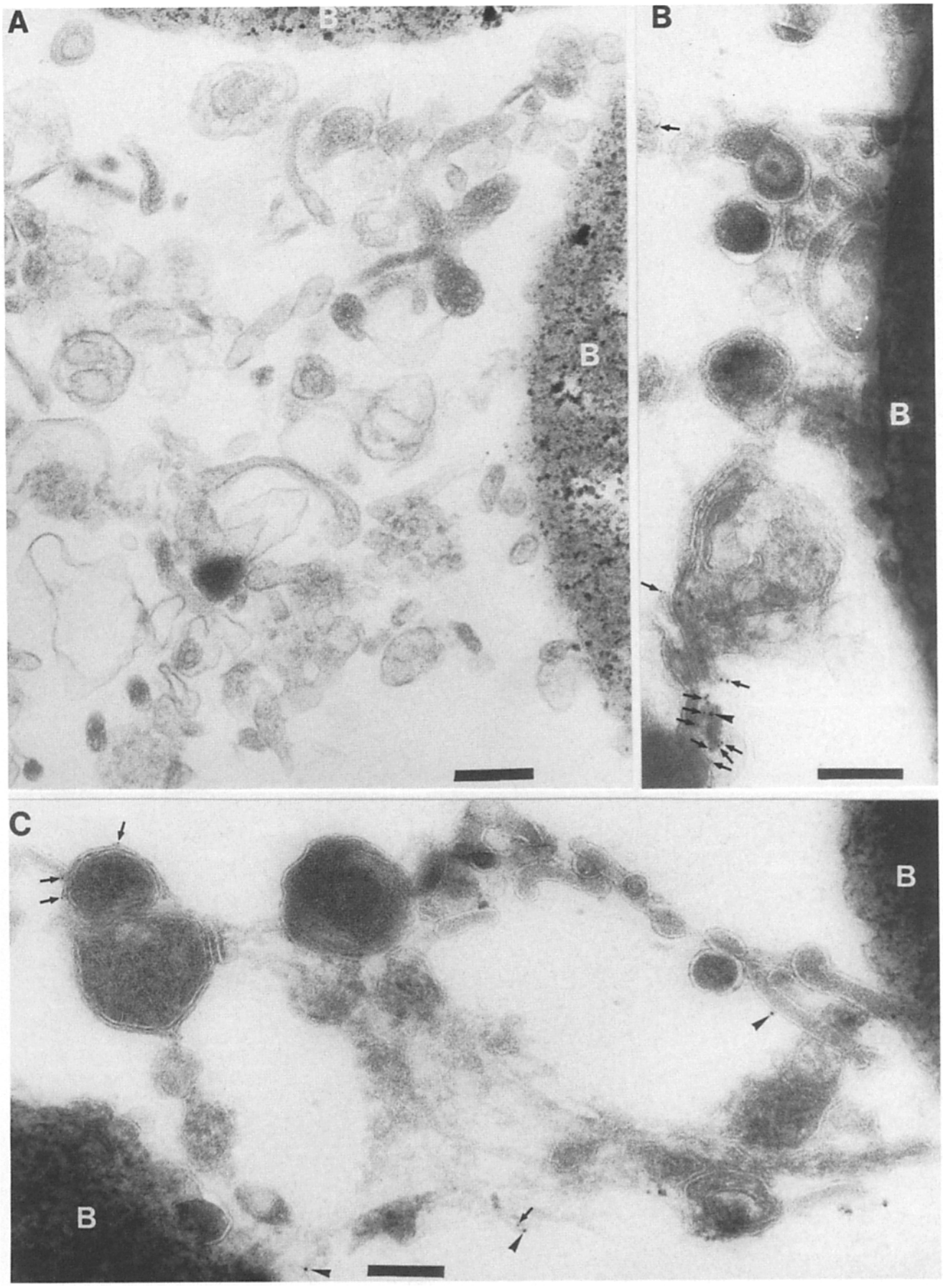


Figure 5. Codistribution of the CI-MPR and the CD-MPR in liver membranes isolated from the PNS fraction. The indicated antibody preparations were conjugated to Dynabeads and used to isolate membranes from bovine liver PNS as described in Materials and Methods. The samples were analyzed for the CI-MPR (▩) and the CD-MPR (▨) by quantitative immunoblotting and the results are expressed as the percentage of each MPR adsorbed on the Dynabeads. The data shown are the mean ± the standard error of the mean of six experiments, three of which used fresh bovine liver PNS in Dynabead wash containing 0.5 M sucrose, and three of which were done on PNS aliquots that had been frozen, using 0.25 M sucrose in the Dynabead wash. There was no significant difference between these two groups. The ratios of CI-MPR to CD-MPR in the membranes isolated on the immune beads (CI/CD) were determined separately in each of the six experiments. The mean values are shown in the inset, expressed relative to the initial PNS fraction (which was taken as one).

The MPR-enriched Membranes Have Biochemical Characteristics Which Are Distinct from Other Intracellular Organelles

To optimize the purification of MPR-containing membranes, various fractions from a sucrose gradient were pooled and used as input fractions for immunoisolation. The samples were then analyzed and compared for maximal adsorption of the CI-MPR and the CD-MPR, and minimal adsorption of protein. Pool IV, corresponding to fractions 7–8 of the gradient profiles illustrated in Fig. 2 and representing a density range of 1.104–1.123 g/ml, showed the highest enrichment in MPR-containing membranes (Fig. 7).

When the immunoadsorbed pool IV membranes were analyzed for the presence of various enzymes of known subcellular distribution, the results shown in Fig. 8 were obtained. The data are presented as the yield (see the legend to Fig. 8) of each respective component through the sucrose gradient and immunoisolation steps in each of two experiments.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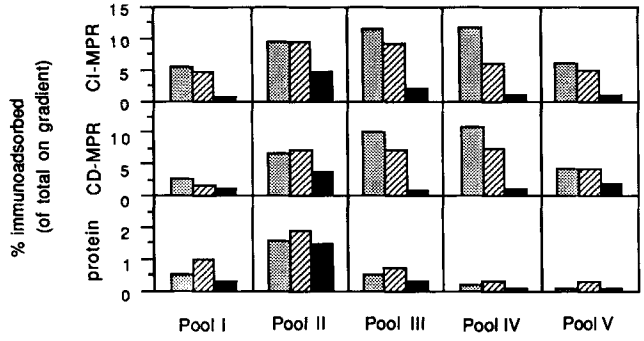

Figure 7. Immunoisolation of membranes enriched in the CI-MPR and CD-MPR after partial purification on a sucrose density gradient. An aliquot of bovine liver PNS was fractionated on a 15–45% linear sucrose gradient as described in Materials and Methods and the legend to Fig. 2. Fractions 1–10 (of 14 total) contained the bulk of the membrane protein and were grouped into five pools for immunoadsorption as follows: *Pool I*, fractions 1–2; *Pool II*, fractions 3–4; *Pool III*, fractions 5–6; *Pool IV*, fractions 7–8; *Pool V*, fractions 9–10. An aliquot of each pool (containing ~0.6 mg membrane protein) was adsorbed with 6 mg of one of the Dynabead preparations: anti-CI-MPR peptide No. 2 (▨), anti-CD-MPR peptide (▩), or nonimmune sera (■). After immunoisolation, the membranes were either analyzed for CI-MPR content (*top row*) or CD-MPR content (*middle row*) by quantitative immunoblotting, or extracted from the Dynabeads with Triton X-100 for protein determination (*bottom row*). The percent immunoadsorption of each component is given relative to the total recovered from the entire sucrose gradient.

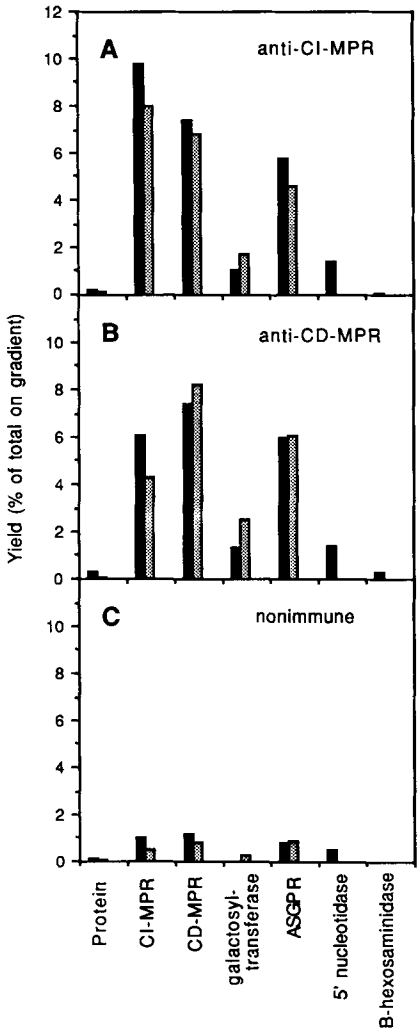

Figure 8. Characterization of marker enzymes in the highly purified CI-MPR- and CD-MPR-enriched membranes. The CI-MPR- and CD-MPR-enriched membranes were isolated from the region of a sucrose gradient corresponding to Pool IV (Fig. 7), adsorbed to Dynabeads, and analyzed for the indicated protein or enzyme activity as described in Materials and Methods. The data are taken from two experiments. In experiment 1 (represented in all panels by ■), a Dynabead to membrane protein ratio of 10 mg/mg was used. In experiment 2 (▨), the ratio was 6 mg/mg. 5′ nucleotidase and β-hexosaminadase activities were not analyzed in experiment 2. (*A*) CI-MPR-enriched membranes, isolated with anti-CI-MPR peptide No. 1 antibodies. (*B*) CD-MPR-enriched membranes, isolated with anti-CD-MPR peptide antibodies. (*C*) Nonimmune background, determined using nonimmune sera coupled to the Dynabeads.

Both anti-MPR bead preparations were substantially enriched (relative to the adsorbed protein) in the CI-MPR, the CD-MPR, and the ASGP R. The latter receptor is known to be distributed primarily between the plasma membrane, endosomes, and the Golgi complex (66). Smaller amounts of galactosyltransferase, 5′ nucleotidase, and β-hexosaminidase were seen. These are generally taken as markers of the Golgi complex, the plasma membrane, and lysosomes, respectively (42, 50). Table I shows the purification of each component (relative to total protein) following the two step procedure. When the Dynabeads were prepared with the anti-CI-MPR antibodies, the purified membranes were 50-fold enriched in the CI-MPR, 42-fold enriched in the CD-MPR, and 31-fold enriched in the ASGP R, with a much lower relative content of the other components. Similarly, the anti-CD-MPR Dynabeads gave preparations which were 50-fold enriched in the CD-MPR, 30-fold enriched in the CI-MPR, and 38-fold enriched in the ASGP R. These data indicate that the MPR-enriched membranes are associated with the endocytic pathway, but are not significantly derived from the Golgi or the plasma membrane.

To examine whether the MPR-enriched membranes are distinct from the TGN as well as the Golgi stacks, a slightly

Figure 6. Immunogold labeling and electron microscopy of immunoisolated membranes. Immunoadsorbed PNS fractions were fixed in 8% paraformaldehyde and embedded in Epon (*A*) or frozen, sectioned, and labeled with antibodies and protein A-gold (*B* and *C*) as described in Materials and Methods. The Dynabeads are identified with a white *B*. (*A*) Membranes isolated with anti-CI-MPR peptide No. 1 beads. (*B*) Cryosections of the membranes adsorbed to the anti-CI-MPR peptide No. 1 beads, double labeled for the CI-MPR (5-nm gold, *arrows*) and the CD-MPR (9-nm gold, *arrowhead*). (*C*) Membranes adsorbed to the anti-CD-MPR peptide beads, double labeled as in *B*. Bars, 200 nm.

*Table I. Overall Purification of Marker Activities after Sucrose Gradient Centrifugation (Pool IV) and Immunoisolation**

Activity	Membranes isolated with	
	Anti-CI-MPR peptides	Anti-CD-MPR peptide
CI-MPR	50 (n = 4, 39–56)	30 (n = 3, 22–39)
CD-MPR	42 (n = 3, 30–52)	50 (n = 2, 26–75)
Galactosyltransferase	7 (n = 3, 4–11)	14 (n = 2, 5–23)
ASGP R	31 (n = 3, 23–39)	38 (n = 2, 21–55)
5′ Nucleotidase	6 (n = 2, 6–7)	5 (n = 1)
β-Hexosaminidase	0.7 (n = 2, 0.4–1.0)	1 (n = 1)

* The fold-purification of each component was calculated as described in Materials and Methods. Mean values are presented, with the number of experiments (n) and the range of values obtained given in parentheses. The starting PNS has a value of one for all components.

broader pool of membranes which contained the bulk of the sialyltransferase activity (a marker for the TGN) was used as the input fraction (Table II). Fractions 6–10 (out of 15 total), representing a density range of 1.087–1.134 g/ml, were pooled and immunoadsorbed with either anti-CI-MPR or anti-CD-MPR Dynabeads. Approximately 45 and 30% of the CI-MPR in the pool was recovered on the anti-CI-MPR or anti-CD-MPR Dynabeads, respectively, along with only 2% of the protein. These same fractions contained ~35% of the CD-MPR, but only 10–13% of the galactosyltransferase activity and 8–9% of the sialyltransferase activity. The relative amount of galactosyltransferase that was co-adsorbed from this pool was slightly higher than that seen from pool IV, but sialyltransferase was no more enriched than galactosyltransferase, supporting our conclusion that the bulk of the MPR-enriched membranes are not derived from the TGN or the Golgi stacks. Only 8% of the succinate-INT reductase activity sedimented in fractions 6–10 of the sucrose gradient, and <10% of this was immunoadsorbed by the anti-MPR Dynabeads, indicating that mitochondria are not being enriched for in this procedure (Table II). Both the galactosyltransferase and the MPRs were somewhat more enriched after immunoisolation from this broader pool of light/medium density membranes, with a greater overall yield (compare Table I and Fig. 8 with Table II).

This pool was also used to evaluate the distribution of acid phosphatase (a lysosomal enzyme) among the immunoadsorbed fractions. There are many different forms of this activity which have been distinguished in a variety of ways, one of which is based on whether they are sensitive or resistant to inhibition by (+)-tartrate (36). The tartrate-resistant form of acid phosphatase found in bovine liver sedimented on a sucrose gradient with a profile similar to that shown for β-hexosaminidase in Fig. 2 (not shown). The profile of the tartrate-sensitive form differed in two ways. First, much less was seen at the top of the gradient (not shown). This would be expected if some of this enzyme was membrane associated, as has been reported for a lysosomal tartrate-sensitive acid phosphatase from human placenta (63). Second, a greater percentage of the tartrate-sensitive form was observed to co-sediment with the membranes of light to medium density (fractions 6–10) which contained the bulk of the Golgi enzymes and the MPRs (Table II). When these membranes (fractions 6–10) were incubated with the anti-MPR Dynabeads, only 3% of the tartrate-resistant acid phosphatase activity was coadsorbed, while 23% of the tartrate-sensitive activity was isolated (Table II). Apparently the tartrate-resistant form of acid phosphatase, like β-hexosaminidase (Fig. 8), has a subcellular distribution which does not significantly overlap with the MPRs. In contrast, a significant portion of the tartrate-sensitive acid phosphatase found in liver appears to be distributed in MPR-enriched membranes.

Polypeptide Composition of the Isolated CI-MPR- and CD-MPR-enriched Membranes

To determine the polypeptide composition of the purified MPR-enriched membranes, the Dynabead fractions were extracted with 1% Triton X-100 after immunoadsorption. Under these conditions, the extracts contain the proteins not directly coupled (via the antibodies) to the beads. Analysis of the extracts by SDS-PAGE and silver staining (Fig. 9) showed that there was significantly more membrane protein on the immune beads compared to the nonimmune beads, as expected from the protein analysis (Fig. 8). The CI-MPR and the CD-MPR are not major bands in any of the lanes, and in fact may not even be detectable, since even after a 50-fold purification they would only represent ~1% of the protein in the extract. The majority of bands in the lanes containing the purified membranes are also seen in roughly the same relative concentrations in the initial PNS or Pool IV fractions. There are, however, several examples of high molecular weight proteins which are significantly enriched in the isolated membranes. Most noticeably, the membranes isolated with the anti-CI-MPR beads are particularly enriched in a 400-kD protein, while the membranes isolated with the anti-CD-MPR beads are strikingly enriched in a 170-kD protein, and to a lesser extent a 185-kD protein (*arrows*, Fig. 9). Each of these proteins is less enriched in the other purified membrane preparation, and is barely detectable or undetectable in the pool IV or PNS fractions. Since the CI-MPR has been localized to clathrin-coated vesicles (8, 53), which contain as the major polypeptide the 180-kD heavy chain of clathrin, it seemed likely the 170- or 185-kD bands might correspond to this protein. There was, however, no enrichment for clathrin heavy chain in any of these fractions, as determined by immunoblotting (data not shown). The identity of these three enriched proteins is, at present, unknown.

*Table II. Yield of Marker Activities after Immunoisolation from the Sucrose Gradient Pool (fractions 6–10)**

Activity (percent total in fractions 6–10)	Membranes isolated with	
	Anti-CI-MPR No. 2	Anti-CD-MPR
	%	%
Protein (15%)	2.3	2.1
CI-MPR (50%)	45	30
CD-MPR (65%)	42	35
Galactosyltransferase (59%)	14	10
Sialyltransferase (61%)	9	8
Succinate-INT reductase (8%)	6	<1
Acid Phosphatase		
Tartrate resistant (15%)	3	3
Tartrate sensitive (28%)	23	23

* Yield refers to the recovery of each component from immunoisolation alone. Details are given in Materials and Methods.

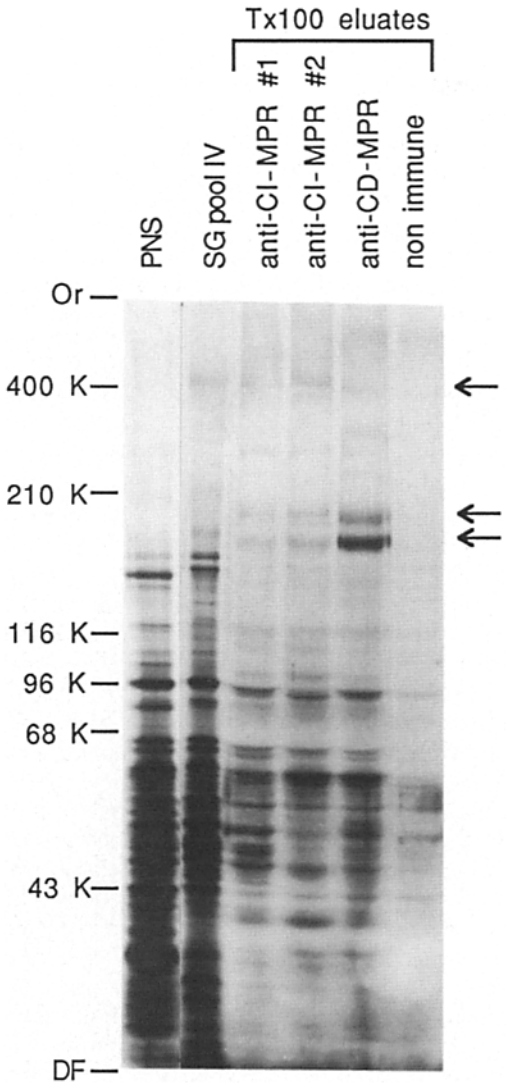

Figure 9. Analysis of highly purified CI-MPR- and CD-MPR-enriched membranes by SDS-PAGE and silver staining. Total PNS membranes were sedimented on a sucrose gradient (*SG*) and the region corresponding to pool IV (see the legend to Fig. 7) was immunoadsorbed with Dynabeads containing either anti-CI-MPR peptide No. 1, anti-CI-MPR peptide No. 2, anti-CD-MPR peptide, or nonimmune antibodies as described in Materials and Methods. The Dynabead fractions were extracted with Triton X-100 and aliquots (5% of the total) were prepared for SDS-PAGE, together with aliquots of the PNS (0.1% of the total) and pool IV (0.2% of the total). After electrophoresis, the 4–12% gradient gel was silver stained; the positions of molecular weight standards are as shown. The arrows indicate the positions of bands which appeared to be particularly enriched in the immunoisolated preparations.

Discussion

The primary goals of this work have been to (*a*) develop a method to specifically purify those membranes from bovine liver which contain the highest densities of CI-MPR and CD-MPR, (*b*) evaluate whether each MPR is enriched in the same, or different populations of membranes, (*c*) determine the most likely cellular organelles from which these membranes are derived, and (*d*) identify other molecules enriched in these organelles to begin to address the fundamental question of how these particular membranes are involved in the targeting and cycling of the MPRs. We have approached these points by taking advantage of the specificity, simplicity, and nondisruptive nature of immunoisolation on magnetic beads. As first described by Howell et al. (10, 35), the use of Dynabeads permits a more gentle isolation procedure than other solid supports such as polyacrylamide beads or formalin fixed *Staphylococcus aureus* cells, since the need for potentially disruptive pelleting and resuspension steps is eliminated. The MPR-containing membranes isolated with this technique are a complex mixture of heterogenous vesicles and tubules (Fig. 6) which appear to be very similar to some of the MPR-containing organelles seen in whole cells (21, 26). This observation supports our belief that we are isolating intact structures with minimal vesiculation and breakage. The immunoisolation step is specific, as judged by our ability to block membrane binding to the Dynabeads with the respective peptides used to generate the antibodies as well as by the low yield of MPRs on the nonimmune Dynabeads. When combined with an initial fractionation on sucrose density gradients, the immunoisolation protocol results in membrane preparations which are enriched ~50-fold in the CI-MPR and the CD-MPR (Table I).

The bulk of the immunoadsorbed membranes are not derived from the Golgi, since there was a much greater enrichment of the CI-MPR and the CD-MPR compared to galactosyltransferase and sialyltransferase (Tables I and II), markers of the *trans*-Golgi cisternae and TGN, respectively (2, 50, 51). There was also an insignificant amount of plasma membrane in these fractions, as judged by the low yield of 5′ nucleotidase (Fig. 8), and no enrichment for mitochondria (2-[*p*-iodophenyl]-3-[*p*-nitrophenyl]-5-phenyltetrazolium reductase) or lysosomes (β-hexosaminidase and tartrate-resistant acid phosphatase). The tartrate-sensitive form of acid phosphatase which sedimented on the sucrose gradients with the membranes of light and medium density was significantly enriched in the purified preparations (Table II). The subcellular distribution of this activity is not known at a quantitative level; it is enriched in lysosomes but is present in other compartments as well (63). It was one of the criteria used by Novikoff and co-workers to identify GERL, a compartment which was originally proposed to include the regions of the Golgi and endoplasmic reticulum responsible for lysosome formation (29). Most of the membranes thought to comprise GERL have more recently been classified as part of the TGN (23). It is interesting, therefore, that a higher percent of the tartrate-sensitive acid phosphatase activity is recovered from the sucrose gradient pool after immunoadsorption than the Golgi marker sialyltransferase (23 vs. 8%, Table II). Apparently the tartrate-sensitive acid phosphatase is not only present in the TGN, but also in the endocytic structures that contain the MPRs, while sialyltransferase is probably confined to the TGN or *trans*-Golgi cisternae.

Most of the CI-MPR found in liver is distributed among endosomes, the Golgi complex, and the plasma membrane (7, 19). The lack of coenrichment of markers for the latter two organelles suggest, therefore, that the membranes we have isolated are endosomes. This is supported more directly by several observations. First, there is a 30–40-fold enrichment for the ASGP R in both the CI-MPR and the CD-MPR membrane preparations (Table I). The ASGP R, a receptor

which functions in the endocytosis of desialylated glycoproteins, is particularly enriched on the plasma membrane and in endosomes (18, 66). Second, the isolated membranes are comprised of a heterogeneous population of vesicles and tubules, which is consistent with the morphological characteristics of endosomes in intact cells or after purification (11, 34, 41, 43, 64). Finally, the total purification (40–60-fold, Table I) and yields (22–23%, Table II) that have been achieved for the CI-MPR and the CD-MPR–enriched membranes using our procedures compare favorably with the values reported for the purification of endosomes by other investigators. Dickson et al. (11) used density gradient centrifugation and size exclusion chromatography to purify vesicles containing newly endocytosed ^{125}I-EGF 23-fold over the PNS fraction prepared from KB carcinoma cells, with a yield of 13%. Hornick et al. (34) also used a density gradient approach to purify vesicles containing endocytosed ^{125}I-LDL from rat liver PNS 73-fold with a yield of 12%. Marsh et al. (41) obtained a 42-fold purification and a 12% yield of endosomes from the PNS fraction of CHO cells using Percoll gradient centrifugation followed by free flow electrophoresis. The procedures we describe are less time consuming than the approaches using multiple density gradients, and they do not require the initial trypsin treatment of the membranes necessary for effective separation by free flow electrophoresis (41), which would probably destroy the cytoplasmic domains of the MPRs. Thus, while we have not established through functional experiments that the MPR-enriched membranes represent endosomes, the composition, morphology, and purification factors of these structures are nearly identical to the vesicles purified by other investigators based on the content of newly endocytosed material.

Immunoisolation of subcellular organelles has been shown to be highly dependent on the surface density of antigen (28), and so it seems likely that we are isolating only those membranes with the highest density of MPRs. The CI-MPR and CD-MPR which can not be isolated may be distributed over a larger surface area of liver membranes at a density below that required for efficient adsorption to the Dynabeads. Both the Golgi stacks and the TGN may fall into this category, as suggested by the lack of coenrichment of galactosyl transferase and sialyltransferase (Fig. 8, Tables I and II). Other factors which may contribute to a immunoisolation yield of <100% include: (*a*) the presence of large, multilamellar structures in which the MPRs are not accessible to the Dynabead-antibody complex, (*b*) partial proteolysis of the MPR cytoplasmic domains, such that the membranes can not be recognized by the anti-peptide antibodies,[3] (*c*) distribution of a fraction of the MPRs on the plasma membrane, which re-seals upon homogenization such that the cytoplasmic domain is inside the vesicles (1, 49), (*d*) the presence of low affinity antibodies in our preparations which can not maintain their attachment during the washing procedures, or limited membrane vesiculation and breakage during the washing procedures, and (*e*) the presence of cellular proteins which bind to the cytoplasmic domains of the MPRs and effectively compete with the antibodies.

3. Immunoblotting experiments with the anti–CI-MPR peptide No. 2 sera show that at least some of the CI-MPR which is not immunoadsorbed does possess an intact cytoplasmic domain. The technique is not sensitive enough to be quantitative, however, and so proteolysis remains a potential contributing factor here.

Two MPR-enriched endosomal compartments have been identified by immunoelectron microscopy which differ in their location within the cell, the relative time required for endocytosed ligands to accumulate within each, and the relative effect of low temperature on the flux of material through each. Geuze et al. (20) have identified a peripheral compartment in both rat liver and Hep G2 cells, termed CURL (for Compartment of Uncoupling of Receptor and Ligand), which is a relatively early endocytic structure that is enriched in both the CI-MPR and the ASGP R. The ASGP R and its ligands are thought to dissociate in these early endosomes (18, 21). The vesicles and tubules which comprise CURL are similar in appearance to the immunoisolated membranes we have obtained. This, together with the high content of ASGP R in the MPR-enriched membranes, suggests that early endosomes probably represent at least a portion of these structures. More recently, a late endosomal compartment has been identified in the Golgi region of NRK cells which is characterized by a very high density of CI-MPR (26). This compartment was shown to be distinct from the Golgi complex, but was enriched in the lysosomal membrane marker LGP 120, suggesting that it is involved in the formation of lysosomes. Furthermore, this late endosomal, prelysosomal compartment is significantly enriched in a tartrate-sensitive form of acid phosphatase. It represents the first acid phosphatase-positive compartment in NRK cells encountered by newly endocytosed ligands on their way to lysosomes (Griffiths, G., manuscript in preparation). The coenrichment of tartrate-sensitive acid phosphatase in the immunoisolated membranes is, therefore, consistent with this prelysosomal compartment being present in our purified preparations. The CI-MPR density of the prelysosomal compartment in bovine liver is probably much lower than that of NRK cells (26) since we did not observe the characteristic intense immunogold labeling seen in the cultured cells. Although the exact relationship of CURL and the prelysosomal compartment is not clear, they do appear to be distinct compartments, and we have most likely enriched for components of both in our purified membranes.

The finding that the CI-MPR and the CD-MPR are coenriched in the same membranes is of particular significance, since it indicates that the two receptors do not sort lysosomal enzymes by entirely different pathways. The membrane preparations isolated by antibodies specific for each receptor are not identical, however. When the sucrose gradient pools were used as the input fraction, the relative ratio of CI-MPR/CD-MPR adsorbed to the beads depended on the antibody used. More CI-MPR was adsorbed to the anti–CI-MPR peptide beads, and more CD-MPR was adsorbed to the anti–CD-MPR peptide beads (Fig. 8 and Table II). Additional differences between the preparations were revealed by analyzing the polypeptide compositions of the MPR-enriched membranes by SDS-PAGE. The relative levels of the 400- and 170-kD bands are markedly different: the former appears to be primarily associated with the CI-MPR–enriched membranes while the latter is most apparent in the CD-MPR–enriched membranes. There are two potential explanations for these differences. First, they could occur if small amounts of each of the two receptors are subsorted to distinct compartments (or distinct regions of the same compartment) which have different polypeptide compositions. This might indicate at least a partial functional difference between the

two MPRs, an idea which has been proposed previously based on differences in the binding specificities of these two proteins (32, 33, 58) as well as the inability to detect CD-MPR-mediated endocytosis (56). The alternative explanation is that these differences reflect relative, rather than absolute, differences in the steady-state distribution of the two receptors. For instance, one of the preparations could be more enriched in early endosomal (CURL) membranes, while the other might contain a relatively higher percentage of membranes derived from late endosomes (including the prelysosomal compartment). This interpretation would be consistent with both MPRs using the same endosomal/prelysosomal structures for delivery of lysosomal enzymes to lysosomes in bovine liver. Further characterization of the purified membranes should clarify this issue, as well as provide additional biochemical information on the basic mechanisms involved in MPR cycling and the segregation of acid hydrolases to lysosomes.

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