Determination of Plasma Membrane Lipid Mass and Composition in Cultured Chinese Hamster Ovary Cells Using High Gradient Magnetic Affinity Chromatography*

(Received for publication, November 16, 1992, and in revised form, December 29, 1992)

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We have utilized wheat germ agglutinin conjugated to iron/dextran particles in conjunction with high gradient magnetic affinity chromatography (HIMAC) to prepare plasma membranes from cultured cells. Membrane-impermeable succinimidyl esters inactivate alkaline phosphodiesterase 1 (APDE-1) and were used to establish the proportion of APDE-1 expressed at the cell surface. The yield of inhibitable APDE-1 provides an accurate indication of plasma membrane yield, which was >90% for Chinese hamster ovary (CHO) cells. Plasma membranes prepared by HIMAC contained <5–13% of endoplasmic reticulum, Golgi, mitochondria, lysosomes, or endosomes. Pulse-chase experiments performed with the α5β1 integrin receptor confirmed the high yield of plasma membrane and demonstrated the utility of this procedure for examining trafficking of proteins to and from the plasma membrane. We determined the lipid content of plasma membranes prepared by HIMAC. CHO plasma membranes contain 49% of total cellular phospholipid, 69% of sphingomyelin, and 64% of cholesterol. Phosphatidylinositol was the only glycerophospholipid highly enriched (71%) in the retained fraction. The glycosphingolipids lactosylceramide and ganglioside GM3 were enriched in the plasma membrane fraction to the same extent as sphingomyelin. The major fraction of the glycosphingolipid precursors glucosylceramide and ceramide was localized to intracellular membranes. These findings indicate that the plasma membrane of CHO cells contains approximately half of the total cellular phospholipids and an even higher percentage of sphingomyelin and cholesterol. The high efficiency and rapidity of this isolation procedure should aid the analysis of plasma membrane components significantly.

The plasma membrane is a dynamic and complex organelle that has been the subject of extensive studies enhancing our understanding of the trafficking of proteins to and from the plasma membrane. Less is known about the lipid components of the plasma membrane and their trafficking. This largely reflects the difficulty of preparing sufficiently pure membranes in high yield, especially from cells in culture. Since lipid components may modulate the activity of receptors and influence trafficking of the plasma membrane, detailed information about the lipid composition of the plasma membrane is essential.

Analyses of the composition and mass of total lipid that comprises the plasma membrane of cultured cells have been controversial. Large and co-workers estimated that 90% of cholesterol and sphingomyelin are in the plasma membrane of cultured fibroblasts and that roughly 50% of the total phospholipid is also present in the plasma membrane (3). Others have suggested that nearly all of the glycosphingolipids are also localized in the plasma membrane. Van Meer has challenged these estimates (1, 2). Based on morphometric determinations of the surface areas of various intracellular compartments and the ratios of their lipid components, he suggested that only 30–35% of sphingomyelin and 25–40% of cholesterol are in the plasma membrane and that the glycosphingolipids are also predominantly intracellular (1, 2). The discrepancies between these estimates have been suggested to reflect unaccounted contributions of endosomal and trans-Golgi membranes to the plasma membrane preparations.

We have now characterized the phospholipid, glycolipid, and cholesterol content of plasma membranes prepared using a ligand-specific isolation procedure that we developed recently (5, 6). Iron/dextran particles (FeDex)1 bearing covalently linked wheat germ agglutinin (WGA/FeDex) are bound to cells, and following disruption of the cells, the plasma membranes are isolated by high gradient magnetic affinity chromatography (HIMAC). Membranes bearing sufficient numbers of FeDex particles are selectively retained by the magnetic field produced by placing steel wool between the

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*The abbreviations used are: FeDex, iron/dextran particles; WGA, wheat germ agglutinin; WGA/FeDex, WGA conjugated to iron/dextran particles; HIMAC, high gradient magnetic affinity chromatography; APDE-1, alkaline phosphodiesterase 1; CHO, Chinese hamster ovary; LacCer, lactosylceramide; GM3, NeuAco2-3Galβ1-4GlcCer; GlcCer, glucosylceramide; sulfo-SHPP, sulfosuccinimidyl 3-(4-hydroxyphenyl)propionate; PBS, phosphate-buffered saline; MeSO, dimethyl sulfoxide; sulfo-NHS-biotin, sulfosuccinimidobiotin; NHS-disulfobiotin, sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate; NBD-cholesterol, N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)-1-aminocaproyl]-b-ethrythro-sphingosine; mAb, monoclonal antibody; FAGE, polyacrylamide gel electrophoresis.

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Plasma Membrane Lipid Mass and Composition in CHO Cells

Poles of a strong (4,000 G) magnet. Membranes that do not bear FeDex particles, individual FeDex particles, and soluble proteins are not retained by the column. The bound membranes are subsequently eluted by removing the column from the magnetic field. We have determined that the yield of plasma membrane can be accurately and conveniently established by selective inactivation of surface APDE-1 with the membrane-impermeant agent sulfo-2-imidyl-3-(4-hydroxyphenyl)propionate (sulfo-SHPP). This procedure for membrane preparation allows multiple plasma membrane components to be measured simultaneously without dependence on the physical-chemical properties of the plasma membrane.

We have documented an 80-90% yield of plasma membrane largely free of endosomes, Golgi, endoplasmic reticulum, and lysosomes as measured by resident enzyme markers and reporter molecules known to pass through these compartments. Our analyses indicate that half of the total cellular phospholipid is present in the plasma membrane of CHO cells. We find that 64-70% of sphingomyelin, cholesterol, and GM1 are also located in the plasma membrane fraction. These values represent some of the first direct measurements of plasma membrane lipid components from cultured cells which do not require large correction factors for yields.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise specified, chemicals and chromatography reagents were purchased from Sigma. Sulfo-NHS-biotin and sulfo-SHPP were from Pierce Chemical Co. The luminol Western membrane preparation allows multiple plasma membrane components to be measured simultaneously without dependence on the physical-chemical properties of the plasma membrane.

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been pelleted at 190,000 g for 2 h in a Beckman SW 55 Ti rotor. Pellets were resuspended in 20% glycerol, 10 mM HEPES, pH 7.0, 0.1% Triton X-100, 5 mM EDTA, protease inhibitor mixture. Each assay of 50 ml contained final concentrations of 1 mM ATP, 40 mM MgCl, 0.03 mM UDP-galactose (specific activity 0.2 mCi/mmol), 100 mM Tris, pH 8.0, 200 mM of dabsylated asialoagalactoglycopeptide, and 0.05% of enzyme buffer. Dabsylated glycopeptide was generated by coupling dabsylchloride to glycopeptide fragments from fetuin as described (17). Sialic acid was removed from the glycopeptide by boiling in 2 N acetic acid for 15 min. Galactose was removed by digestion with diplococcal β-galactosidase in 50 mM cacodylate buffer, pH 6.0. β-Galactosidase activity was determined by measuring the absorbance at 650 nm for 10 min. Incorporation of [3H]galactoside into the acceptor substrate was determined by isolation of the dabsylated glycopeptide product on C-18 Sep-Pak cartridges. Immediately after stopping the galactosyltransferase reaction by dilution with 0.5 ml of ice-cold dH2O, the sample was loaded onto a Sep-Pak cartridge and unincorporated [3H]galactose eluted with 10 ml dH2O. Dabsylated glycopeptide was eluted in 5 ml of 50% methanol, and the amount of [3H]galactoside incorporated was determined by scintillation counting.

Immunoprecipitations and Western Blots—Immunoprecipitations of fibronectin subunits were performed as described (18) except that anti-β1 was used instead of protein A-Sepharose to isolate antibodies, and 1 mg/ml heat denatured bovine serum albumin was present during all immunoprecipitations and rinses. Homogenates were sequentially precipitated with mAb 16, specific for human α5; mAb 33, specific for human and hamster α5; and mAb 66, specific for the β1 subunit (18). The α5 that was precipitated by mAb 16 and the β1 that was brought down by mAb 66 (i.e. not associated with α5) were analyzed separately by SDS-PAGE (18) using a 6% resolving gel and a 3% stacking gel. Proteins for Western blot analysis were resolved by SDS-PAGE using a 10% resolving, 5% stacking polyacrylamide gel. Proteins were transferred to Immobilon-P by standard methods (19). All further incubations and washing were done at room temperature in Tris-buffered saline with 0.2% Tween 20 (TBS-T20). Nonspecific binding was blocked by incubation in 3% bovine serum albumin in TBS-T20, for ≥ 2 h. Incubations with primary antibodies were for 1 h and were followed by three 5-min washes with TBS-T20. Specifically bound antibodies were visualized using horseradish peroxidase-conjugated goat anti-mouse antibody and enhanced chemiluminescence (Amerham Corp.).

Lipid Analysis—Plasma membranes from 10 100-mm dishes were prepared by HIMAC for chemical determination of membrane lipids. Membrane pellets were extracted twice on ice with at least 30 volumes of CHCl3:MeOH, 2:1, v/v, followed by CHCl3:MeOH, 1:2 twice using an Omni Mixer (Ommi International, Norwalk, CT). Total phospholipids were determined by the methods of Gerlach and Deuticke (20). All further incubations and washing were done at room temperature in Tris-buffered saline with 0.2% Tween 20 (TBS-T20). Nonspecific binding was blocked by incubation in 3% bovine serum albumin in TBS-T20, for ≥ 2 h. Incubations with primary antibodies were for 1 h and were followed by three 5-min washes with TBS-T20. Specifically bound antibodies were visualized using horseradish peroxidase-conjugated goat anti-mouse antibody and enhanced chemiluminescence (Amerham Corp.).

Lipid Analysis—Plasma membranes from 10 100-mm dishes were prepared by HIMAC for chemical determination of membrane lipids. Membrane pellets were extracted twice on ice with at least 30 volumes of CHCl3:MeOH, 2:1, v/v, followed by CHCl3:MeOH, 1:2 twice using an Omni Mixer (Ommi International, Norwalk, CT). Total phospholipids were determined by the methods of Gerlach and Deuticke (20). All further incubations and washing were done at room temperature in Tris-buffered saline with 0.2% Tween 20 (TBS-T20). Nonspecific binding was blocked by incubation in 3% bovine serum albumin in TBS-T20, for ≥ 2 h. Incubations with primary antibodies were for 1 h and were followed by three 5-min washes with TBS-T20. Specifically bound antibodies were visualized using horseradish peroxidase-conjugated goat anti-mouse antibody and enhanced chemiluminescence (Amerham Corp.).

Analysis of [3H]palmitate-labeled lipids was performed as follows. The phospholipids of crude lipid extracts were analyzed by two-dimensional thin layer chromatography as described previously (22). High performance TLC plates were activated at 110 °C for 1 h. Labeled samples were applied with cold carrier phospholipids, and development was performed in the first dimension solvent, CHCl3:MeOH:acetic acid, 65:25:10, v/v. After drying the plate for 20 min under a room temperature air stream, the plate was developed in the second dimension solvent, CHCl3:MeOH:88% formic acid, 65:25:10, v/v. The glycosphingolipids from a portion of [3H]palmitate-labeled lipid extracts were purified by the acetylation procedure (23), which involved acetylation overnight at room temperature in pyridine and acetic anhydride. Florisil chromatography, and deacetylation of the glycosphingolipids in 0.15 M sodium methoxide at room temperature for 30 min. Samples were analyzed on high performance TLC plates in the solvent CHCl3:MeOH:H2O, 62:38:0, v/v. The phospholipids were eluted with 99:1, v/v, was used for ceramide quantitation of crude lipid extracts (24). Radiolabeled TLC spots were located using a TLC linear analyzer (EG&G Berthold) and quantitated by scraping into scintillation vials and assaying in the presence of 10 ml of Budget-Solv scintillation fluid.

RESULTS

Isolation of Plasma Membranes from Cultured Cells by HIMAC—Plasma membranes were prepared from cultured cells as described previously (5) by incubating cells with WGA/FeDex at 4 °C, removing unbound WGA/FeDex, disrupting cells, and passing the membranes from the postnuclear supernatant through a column placed between the poles of a 4,000-gauss permanent magnet. The yields of enzyme markers specific for plasma membranes, lysosomes, Golgi, endoplasmic reticulum, and mitochondria in the postnuclear supernatant were similar, falling the range of 65–80% for individual experiments. After washing the membranes in the presence of the magnetic field to remove free WGA/FeDex and membranes not bearing sufficient WGA/FeDex to be retained, the magnetic field was removed, and membranes bearing WGA/FeDex were eluted. The enzyme activities used as markers were quantitatively recovered in the nonretained and/or retained fractions as was observed previously with HepG2 cells (5, 6).

The retained fraction prepared from cultured CHO cells contained 70% of the APDE-1 and 2.7% of the cellular protein (Fig. 1) resulting in a 26-fold enrichment of APDE-1 with respect to total protein. Since APDE-1 is predominantly in the plasma membrane (see below), at least 70% of the plasma membrane from CHO cells was present in the retained fraction. Enzyme markers for intracellular organelles such as lysosomes, mitochondria, and endoplasmic reticulum were recovered at levels of 5–10% of the total loaded onto the HIMAC column (Fig. 1). Thirteen percent of the Golgi marker galactosyltransferase was present in the retained fraction and represented the highest level of a marker not typically associated with the plasma membrane.

The yield of APDE-1 indicated that isolation of plasma membranes from CHO cells using WGA/FeDex and HIMAC

FIG. 1. High yield isolation of plasma membrane from cultured cells by HIMAC. 100-nm diameter culture plates of CHO cells were incubated at 4 °C for 1 h with WGA/FeDex (1 μg/ml) in 5 ml of binding buffer, 1 mg/ml bovine serum albumin. The cells were then washed twice in ice-cold binding buffer to remove unbound WGA/FeDex. Cells from two plates were disrupted, and plasma membrane was isolated by HIMAC as described under "Experimental Procedures." HIMAC nonretained and retained fractions were assayed for enzymatic marker activity specific for plasma membrane (APDE-1), lysosomes (β-hexosaminidase), endoplasmic reticulum (cytochrome c reductase and α-glucosidase II), Golgi (galactosyl transferase), and for protein. The results are presented as the amount of enzyme or protein isolated in the retained fraction as a percent of total recovered from the HIMAC column. The error bars are equal to the standard deviation of 10 separate experiments to determine the average.
was highly efficient. To assure that this was generally true for cell surface proteins, we performed the experiments described in Fig. 2. Cell surface proteins were selectively biotinylated using the membrane-impermeant agent sulfo-NHS-biotin (8). Plasma membranes were then prepared using WGA/FeDex and HIMAC as described above. The membranes in the retained and nonretained fractions were disrupted by freeze-thawing (Fig. 2A) or washing with Na₂CO₃, pH 11 (25) (Fig. 2B), and pelleted at 150,000 × g to remove nonintegral proteins, such as extracellular matrix proteins, which had incorporated biotin. Following SDS-PAGE and transfer to Immobilon, the biotinylated proteins were identified and quantitated by probing with 125I-streptavidin. The membrane impermeant agent sulfo-SHPP we noted that the majority of APDE-1 had been inactivated. This suggested that APDE-1 expressed at the cell surface could be selectively inactivated using membrane-impermeant agents such as sulfo-NHS-biotin, thus providing a means to determine readily and accurately the proportion of APDE-1 expressed at the surface of cultured cells, APDE-1 is inhibited by a number of agents that react with sulphydryl groups such as dithiothreitol, N-succinimidyl 3-(2-pyridyldithio)propionate, N-ethylmaleimide, and dimethyl 3,3′-dithiobispropionimidate (11), suggesting that APDE-1 is inactivated by forming a thioester with sulfo-NHS-biotin. The membrane impermeant agent sulfo-SHPP also inhibited APDE-1 in a concentration-dependent manner, with maximum inhibition being attained at 1 mg/ml sulfo-SHPP (Fig. 3). In the absence of detergent, a maximum of 77% of the cell-associated APDE-1 was sensitive to inhibition by sulfo-SHPP, whereas in the presence of Triton X-100, APDE-1 was completely inactivated. Thus, selective inactivation of APDE-1 at the cell surface using membrane-impermeant agents such as sulfo-SHPP can be used to determine the proportion of cellular APDE-1 expressed at the cell surface. We have found that the proportion of APDE-1 expressed at the cell surface varies considerably among cultured cell lines making it possible to examine the distribution of both APDE-1 essential for establishing the yield of plasma membrane.

In a typical experiment with CHO cells, 77% of the cell-associated APDE-1 was sensitive to inactivation with sulfo-SHPP, i.e. accessible at the cell surface. Seventy-four percent of the cell-associated APDE-1 was present in the retained fraction obtained by HIMAC, indicating that as much as 97% of the accessible APDE-1 (74%/77% × 100) was present in the retained fraction. When plasma membranes were prepared following sulfo-SHPP inactivation of surface APDE-1, only one-fifth of the remaining intracellular APDE-1 activity (equal to 5% of the total cell-associated APDE-1 prior to inactivation) was present in the HIMAC retained fraction. Thus, APDE-1 originating from intracellular membranes accounted for ≤ 5% of APDE-1 in the retained fraction and indicated that the yield of plasma membrane was 92%.

Plasma membranes were isolated from a number of other cell lines to establish the generality of this method for membrane isolation. The plasma cell line MOPC 315P, selected for resistance to prednisone, expresses levels of APDE-1 which are 100-fold greater than the parent cell line (26), making it possible to examine the distribution of both APDE-1.

Plasma Membranes Isolated by HIMAC Are Largely Free of Intracellular Organelles—The use of WGA/FeDex and HIMAC to follow the movement of proteins and other membrane components into and out of the plasma membrane of cultured cells requires minimal contamination with intracellular organelles and endosomes. The data summarized in Fig. 1 show that this condition was met for lysosomes, mitochondria, and endoplasmic reticulum. The amount of galactosyltransferase activity in the retained fraction suggested some Golgi contamination. The extent of contamination with endoplasmic reticulum, Golgi, and endosomal membranes was further addressed by characterizing the synthesis and distribution of the fibronectin receptor (α5β1 integrin) and the transferrin receptor.

Plasma membranes were prepared from CHO K1 cells expressing recombinant human α5 integrin subunit. Following a 20-min labeling period with [35S]Met, the major portion of the integrin α5 subunit is present in the non-

Fig. 4. Inactivation of APDE-1 does not alter its cellular distribution. The effect of sulfo-SHPP treatment on the isolation of plasma membranes by HIMAC was examined using MOPC 315P cells, which express high levels of APDE-1. Cells were harvested from two T25 flasks, pelleted, and washed two times in PBS/C-M. Equal aliquots of cells were incubated in the presence (1 mg/ml) or absence of sulfo-SHPP at 4°C in PBS/C-M, 0.5% MeSO. Plasma membranes were isolated by HIMAC as described under “Experimental Procedures.” Aliquots of HIMAC retained (Ret.) and nonretained (N.R.) membranes were analyzed by SDS-PAGE. The amount of APDE-1 in the retained and nonretained fractions was determined by Western blot analysis using a rabbit antibody to APDE-1, as described by Rebbe et al. (7). Molecular mass standards are: rabbit myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa.

Fig. 5. Intracellular and surface distribution of endogenous integrin β subunits and newly synthesized recombinant integrin α5 expressed in CHO cells. Subconfluent CHO K1 cells, wild type or expressing recombinant human integrin α5, were pulse-labeled with Tran35S-label-labeled methionine and chased in complete medium for the times indicated below the figure. Plasma membranes were then isolated by HIMAC. Aliquots of nonretained and retained fractions from each time point were pelleted for 2 h at 190,000 × g. Pellets were solubilized and subjected to immunoprecipitation of the fibronectin subunits as described in Roberts et al. (18) except that sequential immunoprecipitations were performed on each sample as described under “Experimental Procedures.” Panel A, preintegrin β1 immunoprecipitated with antibody 66 from HIMAC membrane fractions of untransfected CHO K1 cells. Panel B, integrin α5 immunoprecipitated with antibody 33 from nonretained and retained membrane fractions of CHO K1 cells overexpressing human integrin α5.
were addressed by examining the distribution of a fluid phase marker, horseradish peroxidase. After CHO cells were allowed to take up horseradish peroxidase by fluid phase pinocytosis, <10% of the internalized horseradish peroxidase was present in the retained fraction (not shown). Since damage to endosomes could result in release of soluble horseradish peroxidase and an artificially low estimate of the amount of endosomes present in the retained fraction, we also examined the distribution of the transferrin receptor.

The transferrin receptor is constitutively internalized from the plasma membrane to early endosomes through the coated pits and recycled to the plasma membrane (29). As a result 30-40% of the transferrin receptor was present in the retained fraction under conditions in which 75% of the plasma membrane was isolated as calculated from the recovery of the sulfo-SHPP-sensitive APDE-1. At most 40% of the transferrin receptor can be localized to the cell surface based on isolation of the surface membrane using WGA/FeDex and HIMAC, in good agreement with previous estimates using different methods (30). The proportion of transferrin receptor in the plasma membrane fraction stands in marked contrast to that of the fibronectin receptor above. The distribution of the transferrin receptor, like the yield of horseradish peroxidase, indicates that plasma membranes prepared from CHO cells using WGA/FeDex and HIMAC do not contain significant amounts of endosomal membranes.

This conclusion was further substantiated by determining the amount of endosomal transferrin receptor in the retained fraction prepared by HIMAC using a modification of the approach developed by Rodriguez-Boulan and co-workers (8). Transferin receptor on the cell surface was labeled with NHS-disulfobiotin. After allowing the disulfobiotin-labeled transferrin receptor to be internalized, biotin present on transferrin receptor remaining at the cell surface was removed by treatment with glutathione and plasma membranes prepared by HIMAC. The amount of biotinylated transferrin receptor in the retained and nonretained fractions was determined by affinity chromatography on avidin-agarose and quantitative Western blot analysis using a monoclonal antibody specific for the transferrin receptor (Fig. 7). The plasma membrane fraction (retained fraction) contained 10.4 ± 4.9% of endosomal, i.e. internalized, transferrin receptor (average of four separate determinations). We conclude that ≤10% of the endosomes present in CHO cells are found in the plasma membrane fraction prepared by HIMAC.

**Distribution of Lipids in CHO Cells**—The high yield and purity of plasma membranes prepared using WGA/FeDex and HIMAC suggested this would be an ideal method to prepare membranes for determination of their lipid composition. Chemical analysis indicated that the plasma membrane fraction contained 45% of total phospholipid, 64% of total sphingomyelin, and 58% of total cholesterol from CHO cells (Table I). When corrected for the yield of accessible APDE, these values increased to 49% for total phospholipid, 69% for total sphingomyelin, and 64% for total cholesterol. The ratio of cholesterol to phospholipid and sphingomyelin to phospholipid increased 1.6- and 2.2-fold, respectively, in the retained plasma membrane fraction as compared with whole cells (Table I). These data indicate that the plasma membrane of CHO cells represents a major fraction of total membrane bilayer and that the bulk of cholesterol and sphingomyelin resides in the plasma membrane.

Metabolic incorporation of [3H]palmitate makes it possible to examine simultaneously the distribution of sphingomyelin, phospholipids, and glycosphingolipids. After metabolic labeling of CHO cells with [3H]palmitate, 62% of [3H]sphingomyelin was present in the plasma membrane fraction (Fig. 8), a value nearly identical to that obtained by chemical analysis (Table I). Similarly, 43% of the [3H]-labeled total phospholipids were in the retained fraction as compared with 45% determined by chemical analysis (Table I). Phosphatidylycerine was the only glycerophospholipid highly enriched (65%) in the retained fraction. Only 43% of phosphatidylethanolamine was present in the retained fraction, whereas even lower percentages of phosphatidylcholine, the major phospholipid, and phosphatidylinositol were in the retained fraction (Fig. 8).

CHO cells contain a simple pattern of glycosphingolipids (31, 32), namely GlcCer, LacCer, and GcCer. Following metabolic incorporation of [3H]palmitate, GcCer, and LacCer were highly enriched in the retained fraction (Fig. 8). In striking agreement with previous estimates using different methods (30).
contamination with endosomal membranes and other intracellular organelles. We prepared plasma membrane fractions from cultured subconfluent CHO cells that were labeled for 3 h with [3H]palmitate in complete medium. After washing twice with binding buffer, plasma membranes were isolated using WGA/FeDex and HIMAC as described. Equal aliquots of HIMAC retained and nonretained fractions were pelleted as described in Fig. 5. The phospholipids and the glycolipids were analyzed by thin layer chromatography as described under "Experimental Procedures." Percent retained cpm divided by total recovered cpm values from four separate experiments are presented as the mean ± S.E. Total phospholipid is the sum of the five major phospholipids (sphingomyelin (SM), phosphatidycholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylethanolamine (PE)). In contrast, only 40% of GlcCer was present in the retained fraction (Fig. 8). Ceramide, the biosynthetic precursor for both sphingomyelin and the glycosphingolipids, was the least plentiful lipid in the plasma membrane fraction with only 23% in the retained fraction (Fig. 8).

**DISCUSSION**

Investigations of the plasma membrane using cultured cells have been hampered by the difficulty of obtaining highly enriched plasma membranes in good yield. This has been particularly troublesome for the analysis of lipids. Conclusions about the composition and distribution of lipid components in the plasma membrane have in some instances been questioned because of uncertainties about the levels of contamination with endosomal membranes and other intracellular organelles (2). In this study we provide evidence that the combination of WGA/FeDex and HIMAC is able to overcome these difficulties, providing an effective method for the analysis of both protein and lipid components of the plasma membrane. We prepared plasma membrane fractions from cultured cells such as CHO with yields in the range of 80–90% and with low levels of contamination from intracellular membranes including endosomes, endoplasmic reticulum, lysosomes, mitochondria, and Golgi.

The yield and purity of plasma membranes prepared by HIMAC were verified by independent criteria. Although APDE-1 is frequently used as a plasma membrane marker, the proportion of APDE-1 present at the plasma membrane can vary considerably among different cells and under different growth conditions. The proportion of cell-associated APDE-1 expressed at the cell surface can be calculated accurately from its inactivation by membrane-impermeant agents such as sulfo-SHPP. Since only APDE-1 accessible at the cell surface is inactivated by SHPP, the yield of APDE-1 sensitive to inactivation by sulfo-SHPP is indicative of the yield of accessible surface membrane. Yields of plasma membrane from CHO, HepG2, and 3T3 cells were higher from subconfluent, as compared with confluent, cells. The more extensive cell-cell and cell-matrix contacts that develop at confluence may limit the accessibility of selective regions of the plasma membrane to WGA/FeDex but not to SHPP. Plasma membranes can be also isolated from cells grown in suspension. Variations in the yield of plasma membrane do not significantly alter the fold enrichment or the levels of contamination with intracellular membranes of these preparations.

The αβ1 integrin is largely confined to the plasma membrane (27, 28). During synthesis the αβ1 integrin undergoes a number of post-translational modifications making it possible to identify those components that are in the endoplasmic reticulum and Golgi. The transport of newly synthesized αβ1 integrin to the cell surface was examined to determine if HIMAC could be used to establish the kinetics of transport to the plasma membrane and the extent to which endoplasmic reticulum and Golgi membranes were present in the HIMAC retained fraction. Even in the presence of large pools of intracellular precursors, it was possible to detect the arrival of the αβ subunits at the cell surface readily. In addition, little of the synthetic precursors were detected in the retained fraction, indicating low levels of contamination with either endoplasmic reticulum or Golgi. Similarly, based on the yield of intracellular transferrin receptor (Fig. 7) plasma membranes prepared by HIMAC are free of endosomes. Based on both enzyme markers and the yield of en-

**TABLE I**

Table 1: Distribution of lipids

<table>
<thead>
<tr>
<th>Lipid</th>
<th>CHO cells</th>
<th>Ratio CHOL/PL</th>
<th>Ratio CHOL/SM</th>
<th>Ratio SM/PL</th>
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</tbody>
</table>

In nonretained (NR) and retained (Ret) membrane

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Retained</th>
<th>Ratio CHOL/PL</th>
<th>Ratio CHOL/SM</th>
<th>Ratio SM/PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>APDE-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>44.9</td>
<td>0.33</td>
<td>6.9</td>
<td>0.064</td>
</tr>
<tr>
<td>CHOL</td>
<td>57.8</td>
<td>0.59</td>
<td>4.3</td>
<td>0.14</td>
</tr>
<tr>
<td>PL</td>
<td>49.0</td>
<td>(0.02)</td>
<td>(1.5)</td>
<td>(0.02)</td>
</tr>
</tbody>
</table>

* Mean of two separate determinations.

† % retained = Ret/(NR + Ret) × 100, where NR is the nonretained fraction and RET is the retained fraction. Greater than 90% of all markers in the postnuclear supernatant were recovered in nonretained and retained fractions following HIMAC.

‡ The ratio of cholesterol to total phospholipid in the retained fraction was significantly greater than that of the nonretained fraction; p < 0.05.

§ The ratio of sphingomyelin to total phospholipid in the retained fraction was significantly greater than that of the nonretained fraction; p < 0.01.

* Mean and standard error of five separate determinations.
Plasma membranes prepared by HIMAC are highly enriched and free of endoplasmic reticulum, Golgi, mitochondria, and endosomas.

Plasma membranes isolated using WGA/FeDex and HIMAC are particularly suited to the characterization of lipid components and their trafficking to and from the plasma membrane. Earlier studies used isolation procedures that provided yields of 4-20% for the plasma membrane (3, 33-35). The total plasma membrane lipid content is determined in these studies by multiplying the molar ratios of individual lipid components in the isolated plasma membrane fraction by factors ranging from 5 to 25 to correct for yield. The high yields of plasma membrane obtained with WGA/FeDex and HIMAC require the use of correction factors of <1.3. The ratios of cholesterol, sphingomyelin, and total phospholipid for the cells, retained, and nonretained fractions were calculated to permit comparison with these previous studies (Table I).

We obtained a molar ratio of cholesterol to total phospholipid in whole CHO cells of 0.36 (Table I), a value identical to that reported for human fibroblasts (3). This ratio increased an average of 1.6-fold to 0.59 in the retained fractions, indicating enrichment of cholesterol in the retained fraction relative to total phospholipid. In two experiments this ratio reached 0.8 in the retained fraction, a value equal to the maximum reported for plasma membranes purified by other techniques (3, 36). Lange et al. (3) found that the cholesterol to phospholipid ratio varies widely with cell density. Differences in cell density may account for the variations in this ratio which we observed among different experiments (Table I). The ratio of sphingomyelin to total phospholipid increased an average of 2.2-fold, from 0.063 to 0.14, in the retained fraction as compared with the total homogenate of CHO cells (Table I), indicating that sphingomyelin is also enriched in the plasma membrane. The ratio of cholesterol to sphingomyelin was reduced consistently in the retained fraction when compared with whole cells or the nonretained fraction (Table I), suggesting that sphingomyelin is more highly enriched than cholesterol in the plasma membrane.

When the lipid values obtained by chemical analysis of plasma membranes purified using WGA/FeDex and HIMAC were corrected for the yield of accessible APDE, approximately 69% of sphingomyelin, 64% of cholesterol, and 49% of total phospholipid were found in the plasma membrane. These results indicate that in CHO cells the plasma membrane represents a major fraction of the total membrane bilayer and that sphingomyelin and cholesterol are primarily found in the plasma membrane. Lange et al. (3) reported that approximately 90% of sphingomyelin and cholesterol and 50% of total phospholipids are present in the plasma membrane of human fibroblasts (3). Based on susceptibility to cholesterol oxidase Lange and Ramos (37) estimated that 92% of the total cellular cholesterol of CHO cells resides in the plasma membrane. Our estimates of plasma membrane sphingomyelin and cholesterol content are 15-25% lower than those of Lange et al. (3, 37). The lower yield of sphingomyelin and cholesterol in plasma membranes prepared by HIMAC may reflect more efficient separation of endosomes from plasma membranes.

The results of metabolic labeling with [3H]palmitate were consistent with the data obtained by chemical assay. The retained fraction contained 62% of the [3H]sphingomyelin as compared with 64% determined by chemical analysis and 43% of the total [3H]phospholipids as compared with 45% by chemical analysis (compare Fig. 8 with Table I). Phosphatidylserine was the only glycerophospholipid highly enriched in the plasma membrane, in agreement with earlier reports (for review see Ref. 1). The results obtained by metabolic incorporation of [3H]palmitate can therefore be compared directly with those obtained by chemical analysis of lipid components.

The subcellular distribution of glycosphingolipids and the intracellular locations of the glycosyltransferases responsible for their synthesis have only recently been examined (for review see Ref. 1). Information about the intracellular distribution of intermediates involved in synthesis of glycosphingolipids such as GM$_3$ may provide insights about the function of these glycolipids as well as the regulation of their synthesis and transport through the cell. Recent evidence suggests that glucose is added to ceramide on the cytosolic surface of the Golgi (38-41), whereas galactose and sialic acid are added to GlcCer and LacCer, respectively, within the Golgi lumen. LacCer and GM$_3$ are thought to move to the plasma membrane via vesicular traffic using the same pathway and machinery as glycoproteins destined for the cell surface (for review see Ref. 42). The neutral glycosphingolipids LacCer and Forssman have been localized to intracellular membranes in human neutrophils (43) and Madin-Darby canine kidney epithelial cells (44), respectively, raising the possibility that the distribution of glycolipids may not be the same in all cell types.

We found that ceramide and its glycosylated derivatives were not represented equally in the plasma membrane fractions (Fig. 8). The same percentages of GM$_3$ and LacCer, 58 and 61%, respectively, were in the retained plasma membrane fraction as sphingomyelin and phosphatidylserine. When corrected for the yield of APDE-I, this indicated 64% of GM$_3$ and 63% of LacCer were at the plasma membrane. In contrast only 40% of GlcCer and 28% of ceramide, the synthetic precursors of LacCer and GM$_3$, were in the retained fraction. Contamination with intracellular membranes could account for one-third to one-half of the ceramide found in the plasma membrane, indicating that as little as 10-15% of the total ceramide in the cell may be in the plasma membrane of CHO cells. The plasma membrane content of endogenous ceramide and GlcCer has not been addressed previously. The distribution of GlcCer in the plasma membrane differs from that of LacCer and GM$_3$, possibly reflecting conversion of GlcCer to LacCer and GM$_3$ as it passes through the Golgi. Alternatively, GlcCer, which is synthesized on the cytosolic surface of the Golgi, may reach the plasma membrane by a different mechanism than LacCer and GM$_3$, resulting in a different distribution.

The low levels of ceramide in the plasma membrane are particularly striking. Recently, ceramide has been proposed to be an intracellular mediator in the regulation of cell proliferation and differentiation (45, 46). For example, in human leukemia HL-60 cells a neutral sphingomyelinase is activated by extracellular tumor necrosis factor-α, producing an increase in ceramide. The ceramide activates a membrane-associated protein kinase that has been implicated in the mode of action of tumor necrosis factor-α. If HL-60 cells have levels of ceramide in their plasma membrane similar to those we have observed in CHO cells and the increases in ceramide stimulated by tumor necrosis factor-α are confined to the plasma membrane, the increase in ceramide at the plasma membrane may be much greater than appreciated previously and would be consistent with its role as an activator of protein kinase.

The isolation of highly enriched plasma membranes in high yield using WGA/FeDex in conjunction with HIMAC has provided new information about the lipid constituents of the plasma membrane. Sphingomyelin, cholesterol, phosphatidyl-
serine, LacCer, and G_{M1} are found predominantly in the plasma membrane. Furthermore, half of the total cellular phospholipid is present in the plasma membranes of CHO cells, indicating that a major fraction of the lipid bilayer is represented by the plasma membrane. Finally, even though synthetic precursors to G_{M1}, i.e. ceramide and GlcCer, are found in the plasma membrane they are present predominantly on intracellular membranes. The efficiency, rapidity, and simplicity of plasma membrane isolation using HIMAC will make it possible to address such issues as the kinetics of transport of newly synthesized glycoproteins and glycolipids to the cell surface. Such studies will provide insights into the trafficking of lipid components to and from the plasma membrane.

Acknowledgment—We thank James Blomquist for suggestions.

REFERENCES