

Endocytosis of GPI-linked Membrane Folate Receptor- α

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Abstract. GPI-linked membrane folate receptors (MFRs) have been implicated in the receptor-mediated uptake of reduced folate cofactors and folate-based chemotherapeutic drugs. We have studied the biosynthetic transport to and internalization of MFR isoform α in KB-cells. MFR- α was synthesized as a 32-kD protein and converted in a maturely glycosylated 36–38-kD protein 1 h after synthesis. 32-kD MFR- α was completely soluble in Triton X-100 at 0°C. In contrast, only 33% of the 36–38-kD species could be solubilized at these conditions whereas complete solubilization was obtained in Triton X-100 at 37°C or in the presence of saponin at 0°C. Similar solubilization characteristics were found when MFR- α at the plasma membrane was labeled with a crosslinkable ¹²⁵I-labeled photoaffinity-analog of folic acid as a ligand. Triton X-100-insoluble

membrane domains containing MFR- α could be separated from soluble MFR- α on sucrose flotation gradients. Only Triton X-100 soluble MFR- α was internalized from the plasma membrane. The reduced-folate-carrier, an integral membrane protein capable of translocating (anti-)folates across membranes, was completely excluded from the Triton X-100-resistant membrane domains. Internalized MFR- α recycled slowly to the cell surface during which it remained soluble in Triton X-100 at 0°C. Using immunoelectron microscopy, we found MFR- α along the entire endocytic pathway: in clathrin-coated buds and vesicles, and in small and large endosomal vacuoles. In conclusion, our data indicate that a large fraction, if not all, of internalizing MFR- α bypasses caveolae.

5-METHYLTETRAHYDROFOLIC acid is an essential vitamin for the biosynthesis of deoxythymidylic acid, purines and the amino acids methionine and serine (Antony, 1992). For several decades the folate-metabolism has been exploited as a target for chemotherapeutic treatment of a variety of cancers using cytotoxic folate-antagonists such as methotrexate (Bertino, 1993). Transport across membranes of (anti-)folates is one of the critical determinants in their chemotherapeutic effectiveness. Two functionally different systems have been implicated in their cellular uptake: (1) the Reduced-Folate-Carrier (RFC)¹ (Sirotnak, 1985), which probably uses an anion-exchange mechanism to introduce (anti-)folates into the cytosol (Henderson et al., 1986), and (2) Membrane Folate

Receptors (MFRs), which internalize (anti-)folates by a receptor-mediated process. After internalization, folates are retained in the cytoplasm by polyglutamation (McGuire et al., 1980).

The transport kinetics by and affinities for (anti-)folates of both systems differ significantly (Ratnam and Freisheim, 1990; Henderson, 1990; Anthony, 1992). The RFC has a relatively high affinity for reduced folates and the antifolate methotrexate (K_m : 1–10 μ M) but has a relatively poor affinity for the oxidized folate, folic acid (K_m : 200–400 μ M). In contrast, MFRs have high affinities for folic acid (K_d : 0.1–1 nM) and reduced folates (K_d : 1–10 nM) but relatively low affinities for methotrexate (K_d > 100 nM). Both of these transport systems can be expressed separately or complementary to each other in one cell (Jansen et al., 1989; Westerhof et al., 1991, 1993, 1994). The function of such simultaneous expression of both systems in one cell remains unclear. Although the cell and tissue distribution of MFRs (Weitman et al., 1992; Ross et al., 1994) has been documented in greater detail than the RFC (Matherly et al., 1994), both systems are constitutively expressed in many tissues and cell types. Recently two cDNAs have been isolated encoding a protein with RFC-activity

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1. *Abbreviations used in this paper:* GPI, glycosyl-phosphatidyl-inositol; IEF, isoelectric focusing; MFR, membrane folate receptor; MFR- α , membrane folate receptor-isoform α ; RFC, reduced-folate-carrier.

from mouse (Dixon et al., 1994), hamster (Williams et al., 1994), and human (Williams and Flintoff, 1995) cDNA-libraries, predicting that the RFC is a glycosylated integral membrane protein that spans the membrane 12 times.

While the translocation of folates by the RFC occurs directly at the plasma membrane (Sirotnak, 1985), the uptake by MFRs is thought to occur after internalization of the receptors into the cells. Several cDNAs encoding at least three different isoforms of MFRs have been isolated (Ratnam et al., 1989; Elwood, 1989; Lacey et al., 1989; Sadasivan and Rothenberg, 1989; Brigle et al., 1991; Shen et al., 1994), and it has been well established that their products are attached to membranes by a glycosyl-phosphatidylinositol (GPI)-anchor (Lacey et al., 1989; Luhrs and Slomiany, 1989; Alberti et al., 1990). In general, GPI-linked proteins are internalized by cells more slowly than integral membrane receptors and possibly through different internalization pathways (Low, 1989; Lisanti et al., 1990; Bamezai et al., 1992; Keller et al., 1992; Watts and Marsh, 1992). Especially with respect to the uptake of (anti-)folates, Anderson and colleagues have shown that in MA-104 monkey kidney cells MFR- α binds and internalizes folates after which the receptor recycles to the cell surface. Furthermore, their study provided evidence that MFR- α works in tandem with a probenecid-sensitive carrier (Kamen et al., 1988, 1991). Electronmicroscopy in combination with these biochemical data led them to propose a model for the uptake of small molecules such as (anti-)folates, termed potocytosis (Rothberg et al., 1990a; Anderson, 1993a). In this model MFR- α is entirely clustered in caveolae, which were originally described as small, non-clathrin-coated invaginations at the plasma membrane (Palade and Bruns, 1968) and serve to concentrate folates. After sealing of the caveolae, rapid acidification of the caveolar content would cause dissociation of folates from MFR- α after which a putative carrier protein would translocate the folates across the membrane into the cytosol. During this process caveolae remain attached to the plasma membrane. To complete the cycle the caveolae open and expose MFR- α to the extracellular space again. Caveolae differ from clathrin-coated buds and vesicles which pinch off from the plasma membrane after uncoating the fuse with the endosomal system. However, recently it was shown that clustering of MFR- α and other GPI-linked proteins in caveolae could be induced by specific antibodies (Mayor et al., 1994). Since the clustering of MFR- α in caveolae, as reported by Anderson and colleagues, could have been induced by antibody binding, the mechanism by which MFR- α mediates the uptake of (anti-)folates into cells remains to be established. In addition, it has been reported that internalization of MFR- α and other GPI-linked proteins proceeds via clathrin-coated or non-clathrin-coated vesicles, both capable of fusing with the entire endosomal system (Hjelle et al., 1991; Nykjær et al., 1992; Watts and Marsh, 1992; Van Deurs et al., 1993; Sandvig and van Deurs, 1994).

Caveolae are thought to exist in many different cell types (Severs, 1988; Rothberg et al., 1992). The integrity of caveolae depends on the presence of cholesterol (Rothberg et al., 1990b; Cerneus et al., 1993). Caveolae have been shown to contain multiple GPI-linked proteins (Ying et al., 1992), an IP₃-sensitive Ca²⁺-channel (Fujimoto et

al., 1992), and an ATP-sensitive Ca²⁺-pump (Fujimoto, 1993). Caveolin is a major constituent of caveolae (Glenney and Soppet, 1992; Rothberg et al., 1992). VIP21, which was shown to be identical to caveolin (Glenney, 1992), has also been localized to the *trans*-Golgi network and has been proposed to be part of the biosynthetic apical sorting machinery in epithelial cells (Kurzchalia et al., 1992; Dupree et al., 1993). Differential solubilization of GPI-linked proteins in non-ionic detergents (for review see Low, 1989) has also been implicated in their sorting to the apical domain of the plasma membrane in epithelial cells (Brown and Rose, 1992; Cerneus et al., 1993; Garcia et al., 1993). In this model GPI-linked proteins together with glycosphingolipids form membrane domains in the *trans*-Golgi network, which are then targeted to the apical domain of the plasma membrane, as initially proposed by Simons and van Meer (1988). Recently, biochemical methods based on the differential solubilization characteristics in non-ionic detergents such as Triton X-100 have become available to isolate membrane domains, which are enriched in GPI-linked proteins and may represent caveolae (Cinek and Horejsi, 1992; Sargiacomo et al., 1993; Fiedler et al., 1993; Lisanti et al., 1994a; Chang et al., 1994). In addition to GPI-linked proteins isolated Triton X-100-insoluble membrane domains also contained GTP-binding proteins and protein-tyrosine kinases. This has led to the proposition of another function for caveolae, i.e., their involvement in signal transduction (Anderson, 1993a,b; Lisanti et al., 1994b).

In the present study, we have used iodinated crosslinkable photoaffinity analogs of folic acid and the antifolate methotrexate to study the trafficking of functional MFR- α and RFC molecules. We have found correlations between the solubilization characteristics of GPI-linked MFR- α with its biosynthesis, internalization, and recycling. Our results show that MFR- α present in detergent-resistant membrane domains are excluded from internalization. Only a small amount of Triton X-100 soluble MFR- α is internalized by the cells, at least in part via clathrin-coated vesicles, and reaches the endosomal compartments.

Materials and Methods

Cell Culture and Biosynthetic Labeling

KB nasopharyngeal carcinoma, and leukemic CCRF-CEM-7A (Jansen et al., 1990b) cells were cultured in a 5% CO₂ atmosphere in RPMI 1640 without folic acid (GIBCO BRL, Gaithersburg, MD), supplemented with 10% dialyzed FCS and 1 nM of folinic acid ([6S]-5-formyltetrahydrofolate). 24 h before each experiment the folinic acid was removed from the cells by changing the medium. For metabolic labeling, cells were depleted from methionine, pulse labeled at 37°C with 3.7 MBq/ml of [³⁵S]methionine (Tran-S-label, 185 MBq/ml, 40 TBq/mmol, ICN Biomedicals, Inc., CA) for 15 min and chased for the indicated time periods at 37°C in culture medium containing 100 μ M unlabeled methionine (Rijnboutt et al., 1992). The media were prewarmed and equilibrated in a 5% CO₂-atmosphere before use. Next the cells were lysed in PBS containing 1% Triton X-100 and 1 mM PMSF at 0°C and aliquots of the lysates were incubated at 37 or 0°C in the absence or presence of 0.2% saponin. Detergent-insoluble material was subsequently removed by centrifugation at 10,000 g at 4°C. Finally, the lysates were immunoprecipitated for MFR- α and immunoprecipitations were analyzed by separation on SDS-PAGE. Quantitation of the gels was performed using a Phospho-Imager (Molecular Dynamics, Sunnyvale, CA).

Synthesis of Pte-ASA-Lys

N^{α} -Pteroyl- N^{ϵ} -(4'-azidosalicyl)-L-Lysine was prepared by the reaction of N^{α} -[N^{10} -(trifluoroacetyl)pteroyl]-L-Lysine (McAlinden et al., 1991) and 4-azidosalicylic acid succinimyl ester in a molar ratio of 1:1.5 at 16°C in subdued light (Price et al., 1986). The trifluoroacetyl group was removed concurrently by the addition of 40 mM Li_2CO_3 solution. The resulting mixture was acidified using 10% aqueous citric acid and the resulting solid was isolated after lyophilization. The pellet was washed successively with water, ethanol, and diethylether. The fine yellow powder thus obtained was ~70% pure by thin layer chromatography ($R_f = 0.15$) on silica (Kodak 13181) using a mixture of isopropanol, methanol, and concentrated NH_4OH of 3:1:1. A single slower moving impurity ($R_f = 0.06$) was detected which reacted positively with ninhydrine and was assumed to be N^{α} pteroyl-L-Lysine based upon its chromatographic retention time. This material was employed in the subsequent iodination step without further purification. The affinity for Pte-ASA-Lys is similar as for folic acid as demonstrated by equimolar competition for MFR- α of [3H]folic acid binding by Pte-ASA-Lys and Pte-[^{125}I]ASA-Lys binding by folic acid (results not shown).

Photoaffinity Labeling and Endocytosis of MFR- α

Labeling of MFR- α present at the plasma membrane of KB cells was performed either by lactoperoxidase-mediated iodination of the cell surface as described previously (Cerneus et al., 1993) or using a crosslinkable photoaffinity analog of folic acid, pteroyl- N^{ϵ} -(4-azido-5-[^{125}I]iodosalicylyc)-L-lysine (Pte-[^{125}I]ASA-Lys) (Fig. 1). Iodination of 200 μ g Pte-ASA-Lys in 200 μ l dimethylformamide was performed as described by Price et al. (1986, 1987) in subdued red light using 1.5 mCi ^{125}I and five iodobeads (Pierce, Rockford, IL). After 30 min at room temperature the solution was applied to a fluorescent, silica gel TLC sheet which was developed in a mixture of 3:1:1 (vol/vol) isopropanol:methanol: NH_4OH . After 3 h Pte-[^{125}I]ASA-Lys (R_f : 0.4) was identified using short-wave UV light (266 nm), carefully scraped and extracted in methanol. After pelleting the silicagel at 13,000 g, the methanol was evaporated under N_2 and the Pte-[^{125}I]ASA-Lys was dissolved in 200 μ l 160 mM Hepes, 2 mM $MgCl_2$ (pH 7.4). Aliquots were used to determine the concentration (usually 170–210 pmol/ μ l) in a spectrophotometer at 282 nm and the specific activity (usually 2.5–3 nCi/pmol) in a gamma counter.

For endocytosis, the cells were washed three times with RPMI 1640 without folic acid, supplemented with 20 mM Hepes, pH 7.3 (RPMI-Hepes) on ice. To remove extracellularly bound ligands the cells were washed three times on ice with 25 mM Mes, 2 mM $CaCl_2$, 140 mM NaCl, pH 4.5, followed by three times with RPMI-Hepes on ice. Next the cells were incubated in subdued red light in 50 pmol/ml Pte-[^{125}I]ASA-Lys in RPMI-Hepes for 15 min on ice. The cells were washed three times with RPMI-Hepes and irradiated with high intensity 365 nm UV-light (Spec-

troline SB-100/F, Spectronics Corporation, Westbury, NY) to covalently bind the Pte-[^{125}I]ASA-Lys to MFR- α at the cell surface. To remove non-specifically incorporated or noncovalently bound Pte-[^{125}I]ASA-Lys, the cells were washed twice with 25 mM Mes, 2 mM $CaCl_2$, 140 mM NaCl, pH 4.5 containing 1 mg/ml bovine serum albumin (BSA) as a scavenger molecule, followed by three times with RPMI-Hepes on ice. Next the cells were chased for the indicated periods of time at 37°C in RPMI-Hepes, supplemented with 1 nM folic acid. To distinguish internalized MFR- α from MFR- α at the plasma membrane, the cells were washed once with ice-cold RPMI-Hepes, washed twice with ice-cold 25 mM Mes, 2 mM $CaCl_2$, 140 mM NaCl, pH 5.5, and incubated with 50 mU/ml neuraminidase (Boehringer, Mannheim, Germany) in the latter buffer for 1 h on ice. To stop the neuraminidase-mediated desialylation of proteins at the plasma membrane, the cells were washed three times with PBS containing 2 mM EDTA and 1 mM PMSF and lysed in 1% NP-40 in H_2O containing 1 mg/ml sialylated fetuin for 30 min on ice. Aliquots of the lysates were incubated in the absence or in the presence of 0.2% saponin on ice and detergent-insoluble material was removed by centrifugation at 10,000 g at 4°C. Counting aliquots of the lysates and the insoluble pellets showed that in the presence of saponin no counts were found in the pellets, indicating complete solubilization of MFR- α under these conditions. The lysates were analyzed on 1 dimensional isoelectric focussing gels with a linear pH-range from 3.9 to 8.8 as described previously (Rijnboutt et al., 1992) and visualized by autoradiography. Quantitation of SDS-PAGE and IEF gels was performed using a Phospho-Imager (Molecular Dynamics). For IEF gels, neuraminidase-sensitive species of MFR- α were quantitated together and expressed as a percentage of the total.

Analysis of Triton X-100-insoluble Membrane Domains on Sucrose Flotation Gradients

The isolation of Triton X-100-insoluble membrane domains on sucrose flotation gradients was performed using the method described by Fiedler et al. (1993) and Sargiacomo et al. (1993). The cells were labeled with Pte-[^{125}I]ASA-Lys as described above. After photoaffinity labeling, described as above, the cells were kept on ice or incubated at 37°C in RPMI-Hepes. Subsequently, the cells were washed with ice-cold RPMI-Hepes and lysed at 0°C in 1% Triton in 25 mM Tris pH 7.2, 5 mM EDTA, 1 mM PMSF and 140 mM NaCl containing 1% Triton X-100 in the absence or the presence of 0.2% saponin. In case fractions of Percoll gradients were analyzed, the fractions of interest were pooled and vesicles were lysed using Triton X-100 at a final concentration of 1%. Next 2 ml of the lysate were mixed with 2 ml 2.4 M sucrose in 25 mM Tris pH 7.2, 5 mM EDTA, 1 mM PMSF and 140 mM NaCl in an SW41 tube and carefully overlaid with subsequently 6 ml 1.1 M sucrose and 2 ml 0.15 M sucrose in the same buffer (Beckman Instrs., Fullerton, CA). The lysates were fractionated at 100,000 g for 18 h at 4°C and aliquots of the fractions were analyzed using SDS-PAGE.

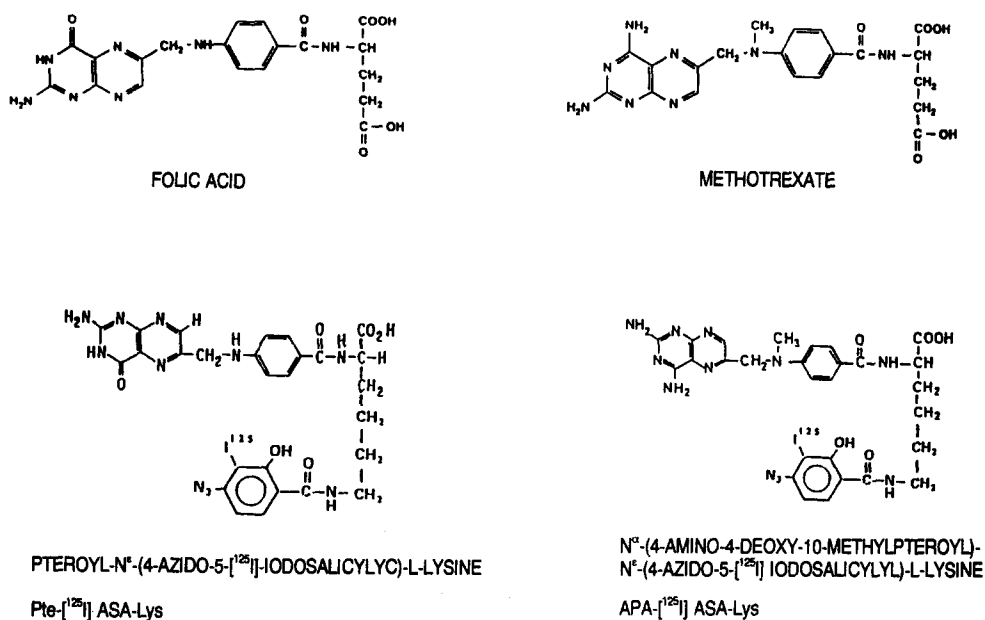


Figure 1. Chemical structures of the two crosslinkable photoaffinity analogs of folic acid and methotrexate, Pte-[^{125}I]ASA-Lys and APA-[^{125}I]ASA-Lys, respectively.

Quantitation of the gels was performed using a Phospho-Imager (Molecular Dynamics). For some experiments (as in Fig. 5), CEM-7A cells were labeled with N^ε-(4-amino-4-deoxy-10-methylpteroyl)-N^ε-(4-azido-5-¹²⁵I-iodosalicylyl)-L-lysine (APA-[¹²⁵I]ASA-Lys), a photoaffinity analog of the antifolate methotrexate constructed for probing the Reduced Folate Carrier (RFC) (Price et al., 1987; Freisheim et al., 1988, 1992) (Fig. 1). Labeling of the RFC at the plasma membrane of 2.10⁷ CEM-7A cells using APA-[¹²⁵I]ASA-Lys was performed in 1 ml 160 mM Hepes, 2 mM MgCl₂ (pH 7.4) for 15 min on ice and followed by irradiation with UV-light.

Recycling of MFR-α and Subcellular Fractionation on Percoll Gradients

To study recycling of internalized MFR-α to the cell surface, KB-cells were labeled with Pte-[¹²⁵I]ASA-Lys as described above and incubated for 2 h at 37°C with RPMI-Hepes, supplemented with 1 nM leucovorin. Next the cells were washed three times with ice-cold RPMI-Hepes followed by two incubations of 10 min with 25 mM Mes, 2 mM CaCl₂, 140 mM NaCl, 1 mg/ml BSA, pH 4.5, to remove all extracellularly bound Pte-[¹²⁵I]ASA-Lys. After washing three times with RPMI-Hepes, the cells were irradiated with UV light as described above and incubated at 37°C for the indicated periods of time. For some experiments (as in Fig. 7 H), the cells were incubated at 37°C before irradiation with UV light, allowing the removal of ligand bound to recycled MFR-α with a final acidic wash. Next the cells were washed once with ice-cold RPMI-Hepes and once with homogenization buffer (0.25 M sucrose, 10 mM Hepes pH 7.2, 1 mM EDTA, and 1 mM PMSF). The cells were homogenized on ice in 0.60-ml homogenization buffer by passing them ten times through a 25-G needle. A post-nuclear supernatant was prepared by centrifugation at 300 g for 10 min at 4°C and 400 μl PNS was fractionated on 7.4 ml 20% Percoll-gradients (Pharmacia Fine Chemicals, Piscataway, N.J.) in homogenization buffer, by centrifugation at 32,000 rpm (100,000 g) for 25 min at 4°C in a Beckman 50Ti rotor. Pte-[¹²⁵I]ASA-Lys-labeled MFR-α was detected in the fractions by counting in a gamma counter.

Ultracryotomy and Immunolabeling

The endocytic pathway was visualized by incubating the cells for 1 h with BSA-Gold (Ø 5 nm, final OD 10). Before fixation the BSA-gold was removed by washing three times with culture medium. Cells were fixed at room temperature by adding 1 ml 4% paraformaldehyde, 0.4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 to 1 ml medium. After 10 min the fixative/medium mixture was replaced by 2% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer. 50 min later the cells were removed from the petri dish using a rubber policeman and transferred to an Eppendorf vial. The cells were spun down and washed three times with PBS, 0.15 M glycine, and finally pelleted in 10% gelatin in phosphate buffer. The gelatin was allowed to solidify, and small cubic blocks were cut at 4°C and infused with 2.3 M sucrose in phosphate buffer for at least 2 h at 4°C. The blocks were mounted on a copper specimen holder and frozen in liquid nitrogen. Ultrathin cryosections were prepared on a Leica Ultracut S (Vienna, Austria) and either single labeled for MFR-α or double labeled (Geuze et al., 1981) for the folate receptor and clathrin (Ahle et al., 1988).

The relative distribution of MFR-α immunoreactive sites was determined by counting gold particles over cell profiles containing a Golgi apparatus and a nucleus. In three different grids 10 profiles were counted. The number of gold particles was measured over the plasma membrane (including coated pits and caveolae), the endosomal apparatus (tubulo-vesicular structures, endosomes, multivesicular endosomes; all containing BSA-gold), the biosynthetic pathway (RER, Golgi apparatus, *trans*-Golgi reticulum), and other vesicles.

Results

Triton X-100 Insolubility of MFR-α during Passage through the Golgi Complex

KB cells, a human nasopharyngeal carcinoma cell line, express large amounts (2–3 × 10⁷ receptors/cell) of MFR-α at their cell surface (McHugh and Cheng, 1979; Antony et al., 1985; Luhrs et al., 1986; Elwood et al., 1986). The protein can be released completely by PI-PLC (results not

shown; Luhrs and Slomiany, 1989) indicating anchoring to membranes by a glycosyl-phosphatidyl-inositol (GPI) anchor (Lacey et al., 1989; Alberti et al., 1990). Several recent reports indicate that GPI-linked proteins become insoluble in the detergent Triton X-100 at 0°C during maturation of the oligosaccharide chains (Cinek and Horejsi, 1992; Brown and Rose, 1992; Dráberová and Dráber, 1993; Cerneus et al., 1993; Garcia et al., 1993). To study the incorporation of MFR-α in Triton X-100-insoluble membrane domains, KB cells were labeled with [³⁵S]methionine and chased after which the cells were lysed in 1% Triton X-100 at 0°C. Aliquots of these lysates were then incubated in the presence or absence of the cholesterol complexing agent saponin and MFR-α was immunoprecipitated and analyzed by SDS-PAGE.

MFR2 was synthesized as a 32-kD precursor which was converted to a 36–38-kD mature species starting after 1 h of chase (Fig. 2 A). This increase in apparent molecular weight is due to the conversion of high-mannose N-linked oligosaccharide chains to the complex type (Luhrs, 1991). The 32-kD precursor of MFR-α remained completely soluble in Triton X-100, whereas after 2 h of chase only 33.7% ± 4.8 (n = 24) of mature MFR-α could be solubilized in Triton X-100 at 0°C. The solubilization of the full complement of mature MFR-α was obtained by the addi-

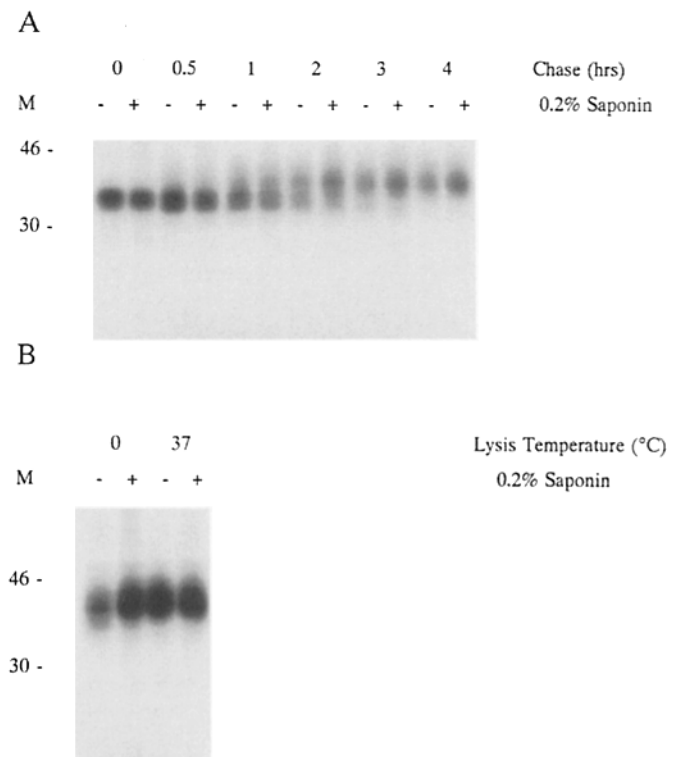


Figure 2. Triton X-100 solubility of biosynthetic species of MFR-α. (A) KB cells were labeled with [³⁵S]methionine, chased for the indicated periods of time and lysed in 1% Triton X-100 in the absence (–) or presence (+) of 0.2% saponin at 0°C. The lysates were immunoprecipitated for MFR-α and analyzed by SDS-PAGE. (B) KB cells were labeled with [³⁵S]methionine, chased for 4 h and lysed as in A at 0°C or at 37°C. Protein-aliquots of the lysates were analyzed on SDS-PAGE. Apparent molecular weight markers are indicated on the left (kD).

tion of 0.2% saponin to the lysate (Fig. 2, A and B) or by incubating the lysate at 37°C (Fig. 2 B). Raising the lysis-temperature or addition of other detergents such as 1% deoxycholate or 1% SDS to the lysates had no effect on the amount of solubilized material (data not shown). Similar solubilization characteristics for MFR- α were also found in other cell types indicating that the effect is independent of the expression level of MFR- α or cell type. Since there was already some 36–38-kD present after 1 h of chase which was completely soluble in Triton X-100 in the absence of saponin (Fig. 2 A), the transfer of MFR- α to its GPI-anchor, as well as the formation of complex-type N-linked oligosaccharides preceded Triton X-100 insolubility.

Both MFR- α Pools Present at the Cell Surface Are Capable of Binding Ligand

Next we examined whether these solubilization characteristics were maintained on the cell surface. To this end, the cell surface of KB cells was iodinated, the cells were lysed as in the previous experiment and MFR- α was immunoprecipitated and analyzed on SDS-PAGE. Fig. 3 shows that 32.8% \pm 3.3 ($n = 6$) of MFR- α was solubilized in Triton X-100 at 0°C and that addition of 0.2% saponin to the lysate resulted in complete solubilization. To determine whether all MFR- α present at the cell surface was capable of binding ligand, bound ligands were removed by an acid wash and the cells were incubated with Pte-[¹²⁵I]ASA-Lys (Fig. 1), an iodinated crosslinkable photoaffinity analog of folic acid. After binding, the excess of ligand was removed by extensive washing, cross-linking was induced by irradiation with UV-light and the cells were lysed. Analysis of the lysates on SDS-PAGE shows (Fig. 3) that Pte-[¹²⁵I]ASA-Lys-labeled MFR- α migrated as a single band of 36–38-kD. Solubilization characteristics similar to [¹²⁵I]-lactoper-

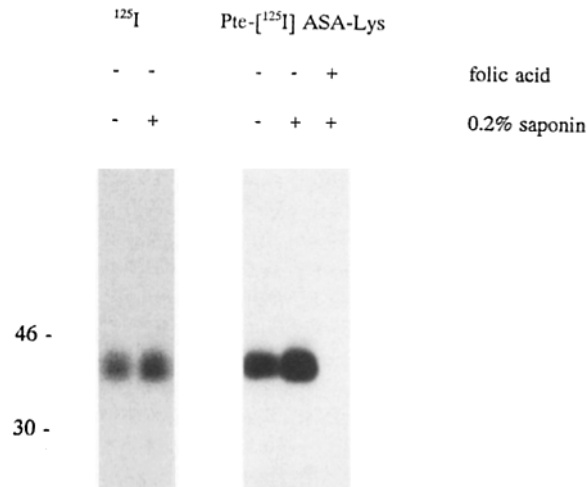


Figure 3. Triton X-100 solubility MFR- α at the cell surface. The cell surface of KB cells was either iodinated followed by immunoprecipitation for MFR- α (¹²⁵I) or KB cells were labeled with Pte-[¹²⁵I]ASA-Lys in the presence (+) or absence (-) of 5 μ M folic acid and lysed as in Fig. 2 A. The immunoprecipitates of ¹²⁵I-labeled cells and aliquots of the lysates of the cells labeled with Pte-[¹²⁵I]ASA-Lys were analyzed on SDS-PAGE. Apparent molecular weight markers are indicated on the left (kD).

oxidase and [³⁵S]methionine (Fig. 2)-labeled MFR- α were found (data not shown). Binding of Pte-[¹²⁵I]ASA-Lys to the cells could be completely prevented in the presence of 5 μ M folic acid. Taken together, these data show that the plasma membrane MFR- α is present in two pools, a Triton X-100 soluble and insoluble pool. Both of these MFR- α pools bind Pte-[¹²⁵I]ASA-Lys with a high specificity. The insolubility is acquired during passage of the Golgi-complex, possibly by incorporation into Triton X-100-insoluble membrane domains (Simons and Van Meer, 1988; Brown and Rose, 1992).

Using sucrose flotation gradients, Fiedler et al. (1993) and Sargiacomo et al. (1993) have reported that such Triton X-100-insoluble membrane domains were highly enriched in GPI-linked proteins and caveolin and probably represent caveolae. We performed an analogous experiment using Pte-[¹²⁵I]ASA-Lys as a ligand for MFR- α to determine whether Triton X-100 insoluble MFR- α in KB cells was present in similar membrane domains. When cells were lysed in Triton X-100 65–70% of MFR- α was found on top of a sucrose flotation gradient (fractions 9–11), whereas the soluble fraction remained at the bottom of the gradient (fractions 1–5; Fig. 4 A). This shows that the insol-

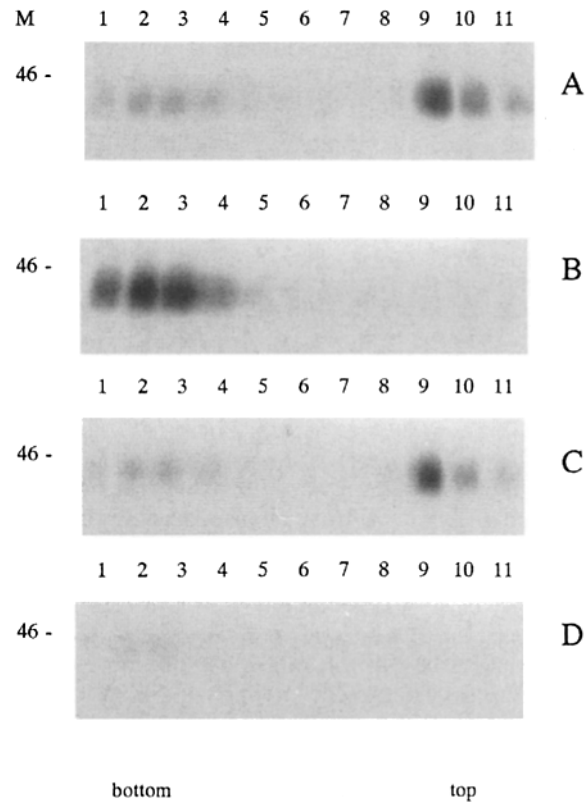


Figure 4. Isolation of Triton X-100-insoluble membrane domains containing MFR- α . KB cells were labeled with Pte-[¹²⁵I]ASA-Lys and lysed in Triton X-100 in the absence (A, C, and D) or presence (B) of 0.2% saponin. The lysates were fractionated on sucrose flotation gradients as described in Materials and Methods and aliquots of the fractions were analyzed on SDS-PAGE. In C and D the cells were allowed to internalize MFR- α labeled with Pte-[¹²⁵I]ASA-Lys after which the cells were D or were not C washed with an acidic MES-buffer to remove noninternalized ligand.

uble fraction of MFR- α was present in Triton X-100-insoluble plasma membrane domains. After addition of 0.2% saponin during lysis of the cells MFR- α was recovered completely at the bottom of the gradient (Fig. 4 B), showing that removal of cholesterol interferes with their integrity, as was previously suggested for alkaline phosphatase in the human chorion carcinoma cell line BeWo (Cerneus et al., 1993).

The Reduced Folate Carrier Is Absent from Triton X-100-insoluble Microdomains

Rothberg et al. (1990) have proposed that the uptake of folates by MFR- α takes place in caveolae in a process which has been termed potocytosis. During this process MFR- α concentrates folates in caveolae after which the folates are translocated to the cytosol by an as yet unidentified carrier protein. A good candidate protein to fulfill this role is the Reduced Folate Carrier (RFC), which has been implicated in (anti-)folate translocation across the plasma membrane and is thought to be an integral membrane protein (Dixon et al., 1994; Williams et al., 1994). Since the distribution of MFR- α on sucrose flotation gradients was identical to the characteristic profile reported for GPI-linked proteins and a few integral membrane proteins (Fiedler et al., 1993; Sargiacomo et al., 1993; Lisanti et al., 1994a), we examined whether the RFC was present in Triton X-100-insoluble plasma membrane domains. To visualize the RFC, a similar cross-linkable photoaffinity folate analog was used. This photoprobe (APA- ^{125}I]ASA-Lys; Fig. 1) was based on the antifolate methotrexate, for which the RFC has a high affinity (Jansen et al., 1990b). Based on kinetic studies with (anti-)folates having preferential affinities for either MFR- α or the RFC, KB cells have been shown to functionally express both MFR- α and the RFC simultaneously, although the RFC is expressed in limited qualitative amounts in KB cells (Jansen et al., 1990a; Westerhof et al., 1991, 1993, 1994). Therefore, APA- ^{125}I]ASA-Lys was used to label the RFC in CEM-7A cells, a previously isolated subline of human leukemia CCRF-CEM cells overexpressing the RFC more than 10-fold (Jansen et al., 1990b). Next the cells were lysed in Triton X-100 at 0°C and run on sucrose flotation gradients. A diffuse band of 70–90 kD indicative for the RFC was detected on SDS-PAGE as shown in previous studies (Freisheim et al., 1988, 1992) and found on the bottom of the sucrose gradient (Fig. 5). We conclude that the RFC is excluded from Triton X-100-insoluble plasma membrane domains. If these domains represent caveolae, RFC cannot function in tandem with MFR- α during potocytosis of folates (Kamen et al., 1991). MFR- α was not detected in this experiment because CEM-7A cells do not express folate receptors. However, when APA- ^{125}I]ASA-Lys was used to label KB cells, the photoprobe also specifically labeled MFR- α without changing its distribution on the sucrose gradient (data not shown). In another control experiment in which cell surface-iodinated KB cells as well as CEM-7A cells were lysed in 1% Triton X-100 and run on sucrose flotation gradients, a small subset of iodinated membrane proteins were found on top of the gradients whereas the bulk of iodinated material was found on the

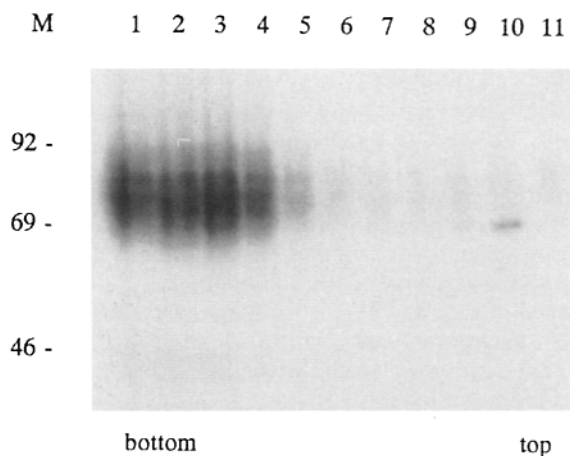


Figure 5. The reduced folate carrier is absent from Triton X-100-insoluble membrane domains. CEM-7A cells, which overexpress the reduced folate carrier were labeled using APA- ^{125}I]ASA-Lys and lysed in Triton X-100 at 0°C. The lysate was fractionated on sucrose flotation gradients as described in Materials and Methods and aliquots of the fractions were analyzed on SDS-PAGE. The 69-kD band in lane 10 has been identified to be serum albumin, which has some nonspecific binding capacity for APA- ^{125}I]ASA-Lys.

bottom, showing the specificity of the flotation procedure (data not shown).

Only Triton X-100-soluble MFR- α Is Internalized by KB Cells

Next we studied the internalization of MFR- α in KB cells. After removing bound ligands by an acidic wash, the cells were labeled with Pte- ^{125}I]ASA-Lys, washed and irradiated with UV-light to covalently link the ligand to MFR- α . The cells were allowed to internalize MFR- α for the indicated periods of time, washed on ice and treated with neuraminidase to distinguish internalized from noninternalized MFR- α . Next the cells were lysed in Triton X-100 in the absence or the presence of 0.2% saponin and the lysates were applied to isoelectric focussing (IEF) gels.

The results are shown in Fig. 6. Again only 33% of MFR- α was solubilized in the absence of saponin. Without chase, treatment of the cells with neuraminidase resulted in a complete shift of all isoforms of MFR- α to higher isoelectric points. After 1 h of chase a maximum of $12.3\% \pm 2.8$ ($n = 3$) MFR- α had become resistant to the neuraminidase treatment, indicating these molecules had been internalized during incubation of the cells at 37°C. The amount of internalized MFR- α was independent of the presence of saponin during lysis of the cells, showing that endocytosis was limited to the MFR- α molecules soluble in Triton X-100 in the absence of saponin. We conclude that MFR- α present in Triton X-100-insoluble membrane domains did not participate in the internalization. When the protocol of the experiment was changed by cross-linking Pte- ^{125}I]ASA-Lys with UV-light after the internalization of MFR- α at 37°C identical results were obtained, as was the case when lactoperoxidase-mediated cell surface-iodination of the cells followed by immunoprecipitation of MFR- α was used, showing that neither absence nor cross-linking of the ligand to MFR- α interferes with its trafficking (data not

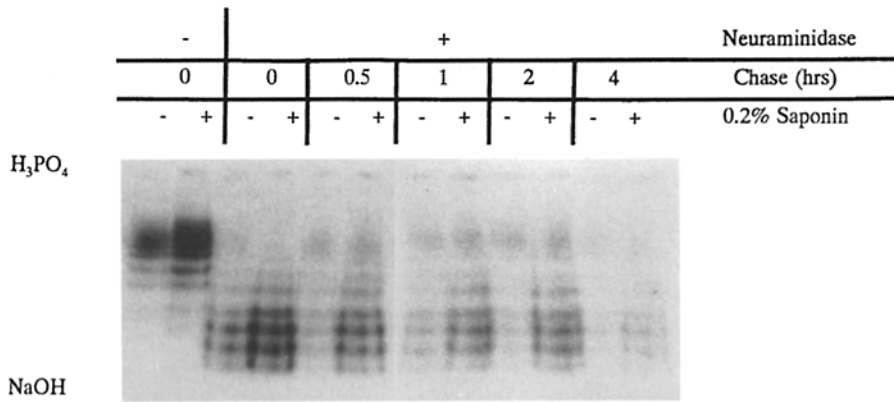


Figure 6. Endocytosis of MFR- α . KB cells were labeled with Pte-[¹²⁵I]ASA-Lys and irradiated with UV-light to covalently link the ligand to MFR- α . Next the cells were allowed to internalize MFR- α for the indicated periods of time (*hours*), washed on ice and treated with (+) or without (-) neuraminidase to distinguish internalized and noninternalized MFR- α . The cells were lysed as in Triton X-100 in the absence (-) or presence (+) of 0.2% saponin and the lysates were applied to isoelectric focussing gels with a linear pH-range from 3.9 to 8.8.

shown). In a separate experiment in which cells labeled with Pte-[¹²⁵I]ASA-Lys were allowed to internalize MFR- α at 37°C for 4 h and subsequently analyzed on sucrose flotation gradients these data were confirmed (Fig. 4, C and D). When extracellularly bound ligands were removed by an acidic wash of the cells after the incubation at 37°C, only a small amount of MFR- α was found at the bottom of the gradient (Fig. 4 D), again showing that internalized MFR- α was soluble in Triton X-100.

Internalized MFR- α Recycles to the Plasma Membrane

Recycling of MFR- α was studied by cell-fractionation on Percoll density gradients. KB cells were labeled with Pte-[¹²⁵I]ASA-Lys as above, washed and allowed to internalize MFR- α at 37°C for 2 h. Due to the high affinity of folic acid/Pte-[¹²⁵I]ASA-Lys for MFR- α intracellular dissociation of Pte-[¹²⁵I]ASA-Lys from MFR- α during endocytosis is thought to be negligible (Kamen et al., 1988). Subsequently noninternalized ligand was removed by an acidic wash and internalized ligand was cross-linked to the receptor at 0°C by irradiation of the cells with UV-light. Intracellular Pte-[¹²⁵I]ASA-Lys-labeled MFR- α was allowed to recycle at 37°C for the indicated periods of time. Postnuclear supernatants were prepared and fractionated on Percoll gradients.

Fig. 7 A shows the distribution of total cell associated Pte-[¹²⁵I]ASA-Lys-labeled MFR- α after binding and internalization of Pte-[¹²⁵I]ASA-Lys (*open squares*). After surface-associated Pte-[¹²⁵I]ASA-Lys had been removed by an acidic wash, the internalized MFR- α distributed at steady state in two overlapping peaks (fractions 3 and 5; *solid squares*). Subtraction of these curves showed that the plasma membrane fractionated in the middle of the gradient (fractions 5-6-7). Fig. 7, B-G show that during the incubation of cells at 37°C, internalized MFR- α was slowly but progressively transported from the intracellular pool in fraction 3 to the plasma membrane in fraction 6 (*solid squares*), when compared to the intracellular distribution at steady state (*open squares*). In a parallel experiment in which the irradiation of the cells with UV-light was performed after the chase incubation at 37°C (i.e., after recycling), identical distributions for recycling MFR- α were found (data not shown). This again indicated that cross-linking of Pte-[¹²⁵I]ASA-Lys to MFR- α did not interfere with its intracellular transport. However, this procedure

allowed the release of ligands bound to recycled MFR- α by an acidic wash after the chase period (Fig. 7 H). It shows that part of MFR- α had recycled to the plasma membrane. However, the majority of internalized, Pte-[¹²⁵I]ASA-Lys-labeled MFR- α was retained in intracellular compartments (Fig. 7 H) and was not triggered to recycle to the plasma membrane by longer chase periods (not shown). We conclude that part of the internalized MFR- α recycles slowly to the plasma membrane whereas the majority remains in intracellular compartments and is presumably degraded.

Since previous experiments had shown that only Triton X-100 soluble MFR- α participated in endocytosis, we determined the Triton X-100 solubility of recycling MFR- α on sucrose flotation gradients at all chase times used in Fig. 7. Therefore, Triton X-100 was added to fractions 2-3-4 (Fig. 8, A and C) or fractions 6-7-8 (Fig. 8, B and D) of the Percoll gradients to a final concentration of 1% and the lysates of the pooled fractions were applied to sucrose flotation gradients as the cell lysates in Fig. 4. The results of two of these experiments are shown in Fig. 8. After a chase of 5 min (Fig. 8, A and B) and after a chase of 60 min (Fig. 8, C and D), as well as at the other periods of time (not shown), more than 90% of MFR- α was recovered at the bottom of the gradient. This indicates that MFR- α remained soluble in Triton X-100 during its recycling to the plasma membrane and that movement of MFR- α from the Triton X-100 soluble pool to the Triton X-100 insoluble pool only occurred very slowly.

Immunoelectron Microscopy

Finally, the intracellular distribution of MFR- α in KB cells was examined using immunolocalization in the electron microscope. The cells were fixed and ultrathin cryosections were immunolabeled for MFR- α . Fig. 9 A shows abundant homogeneous labeling for MFR- α at the plasma membrane and in budding vesicles. The relative distribution of MFR- α immunoreactive sites (Table I) showed that almost 90% of the receptor is present at the cell surface. Based on morphological criteria, caveolae were detected only incidentally in KB cells. Most of the budding vesicles at the plasma membrane were clathrin-coated pits, which colabeled for MFR- α (Fig. 9 B). 9.3% of MFR- α was also found in a variety of large and small endosomes (Fig. 9, C and D). Only 2% of the MFR- α immunoreactive

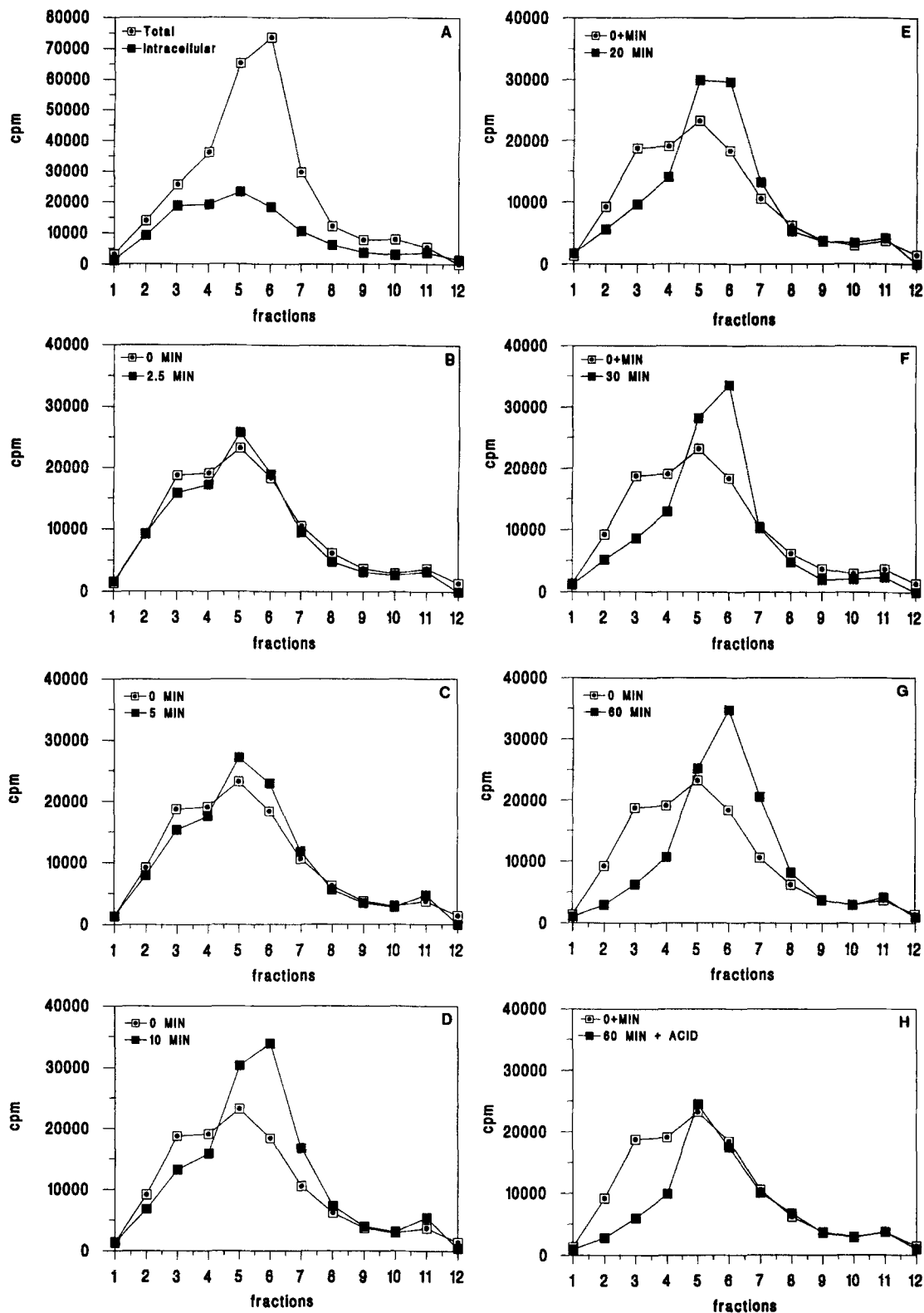


Figure 7. Recycling of internalized MFR- α to the plasma membrane. KB cells were labeled with Pte-[125 I]ASA-Lys and allowed to internalize MFR- α at 37°C. Noninternalized MFR- α was stripped by an acid wash, the cells were irradiated with UV-light to covalently link internalized ligand to MFR- α and chased for the indicated periods of time (*minutes*). Next a postnuclear supernatant was fractionated on Percoll gradients and fractions were counted in a Gamma counter. (A) Distribution of total MFR- α and the distribution of internalized MFR- α after an acid wash. (B-G) Time course of changes in the distribution of intracellular and recycling MFR- α (filled squares) as compared to the distribution before the chase (open squares). (H) Distribution of intracellular MFR- α before (open squares) and after (filled squares) the chase period. In this case the irradiation with UV light was performed after a final acid wash at the end of the chase period.

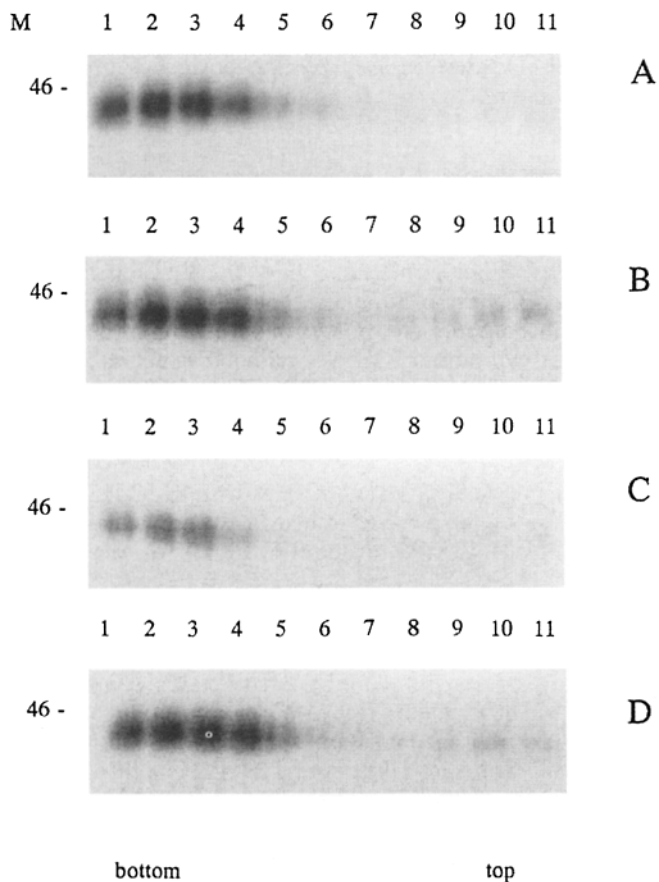


Figure 8. Analysis of recycling MFR- α on sucrose flotation gradients. Top and bottom fractions of Percoll gradients shown in Fig. 7 C (chase = 5 min) and Fig. 7 G (chase = 60 min) were pooled. The pooled fractions were lysed in 1% Triton X-100 and subjected to sucrose flotation gradient centrifugation. Distributions were visualized by analysis on SDS-PAGE. (A) Bottom fractions 2-3-4; chase = 5 min. (B) Top fractions 6-7-8; chase = 5 min. (C) Bottom fractions 2-3-4; chase = 60 min. (D) Top fractions 6-7-8; chase = 60 min.

sites was found along the biosynthetic pathway (RER and Golgi complex) (Fig. 9 E).

Taken together, the present study provides evidence that only small amounts of MFR- α are slowly internalized and recycled to the plasma membrane by KB cells. Internalized MFR- α has access to the entire endocytic apparatus, probably after internalization by clathrin-coated vesicles. MFR- α present in large Triton X-100-insoluble membrane domains remain at the plasma membrane.

Discussion

In the present study the biosynthesis and endocytosis of MFR- α and its recycling to the plasma membrane were studied with respect to its partial insolubility in the detergent Triton X-100, as found for other GPI-linked proteins (Cinek and Horejsi, 1992; Brown and Rose, 1992; Sargiacomo et al., 1993; Fiedler et al., 1993; Chang et al., 1994). We found that 65–70% of MFR- α becomes insoluble during biosynthetic transport to the plasma membrane, probably in the Golgi complex. At the plasma membrane only

soluble MFR- α participates in endocytosis. Internalized MFR- α remains soluble in Triton X-100, also after MFR- α has recycled to the plasma membrane. Internalization of MFR- α and its recycling to the plasma membrane were limited to only 10–15% of MFR- α present at the cell surface. This is in good agreement with the quantitative immunoelectron microscopy, in which 87% of the MFR- α was found at the cell surface and only 10% in the endocytic compartments.

The molecular basis for the distribution of GPI-linked proteins in two pools characterized by their differential solubilization in detergents such as Triton X-100 is not well understood. Intramolecular differences of GPI-linked proteins present in either one of these pools have not been detected. This study shows that the distribution over these two pools is independent of the binding of ligand to GPI-linked MFR- α . As several groups have already indicated, we conclude that the microenvironment of these proteins must be responsible for the solubilization characteristics of these proteins. This was indicated by the complete solubilization of MFR- α after the addition of the cholesterol complexing agent saponin to the Triton X-100, indicating a key role for cholesterol in the integrity of insoluble membrane domains (Rothberg et al., 1990; Brown and Rose, 1992; Cerneus et al., 1993). Glycosphingolipids have also been suggested to be a critical constituent of Triton X-100-insoluble microdomains (Brown and Rose, 1992). The homogeneous labeling for MFR- α at the plasma membrane as detected in our immunoelectronmicroscopy, suggested the presence of small rather than large insoluble membrane domains. The possibility that the formation of large insoluble domains is induced during solubilization in Triton X-100 by clustering of individual GPI-linked proteins or small insoluble domains cannot be excluded. Since only part of the GPI-linked proteins becomes trapped in these domains, the fact remains that there must still be a difference leading to the differential solubilization. These biochemically defined microdomains share many characteristics of the morphologically defined caveolae, however, differences in composition due to the methodology used cannot be excluded.

Almost 90% of total cell-associated MFR- α was homogeneously distributed at the plasma membrane. MFR- α was found to colocalize with clathrin in coated pits. A similar localization has been observed for the GPI-linked $\alpha 2$ -macroglobulin receptor (Nykjær et al., 1992). In addition, neuroblastoma cells lacking caveolae and caveolin have been reported to internalize a glycolipid-anchored prion protein via clathrin-coated pits and vesicles (Shyng et al., 1994). This is in contrast with the observation that GPI-linked proteins are excluded from clathrin-coated pits (Lemansky et al., 1990). MFR- α was also detected along the entire endocytic pathway using immunoelectronmicroscopy. These observations are in contrast with the localization of MFR- α described by Rothberg et al. (1990) in MA104 monkey kidney cells. These authors found MFR- α uniquely in large clusters in caveolae at the plasma membrane. Such large clusters of MFR- α in caveolae were not detected in prefixed KB cells. On the other hand, cell surface immunolabeling of unfixed KB cells resulted in large clusters of MFR- α at the plasma membrane, which only occasionally colocalized with caveolae (data not shown).

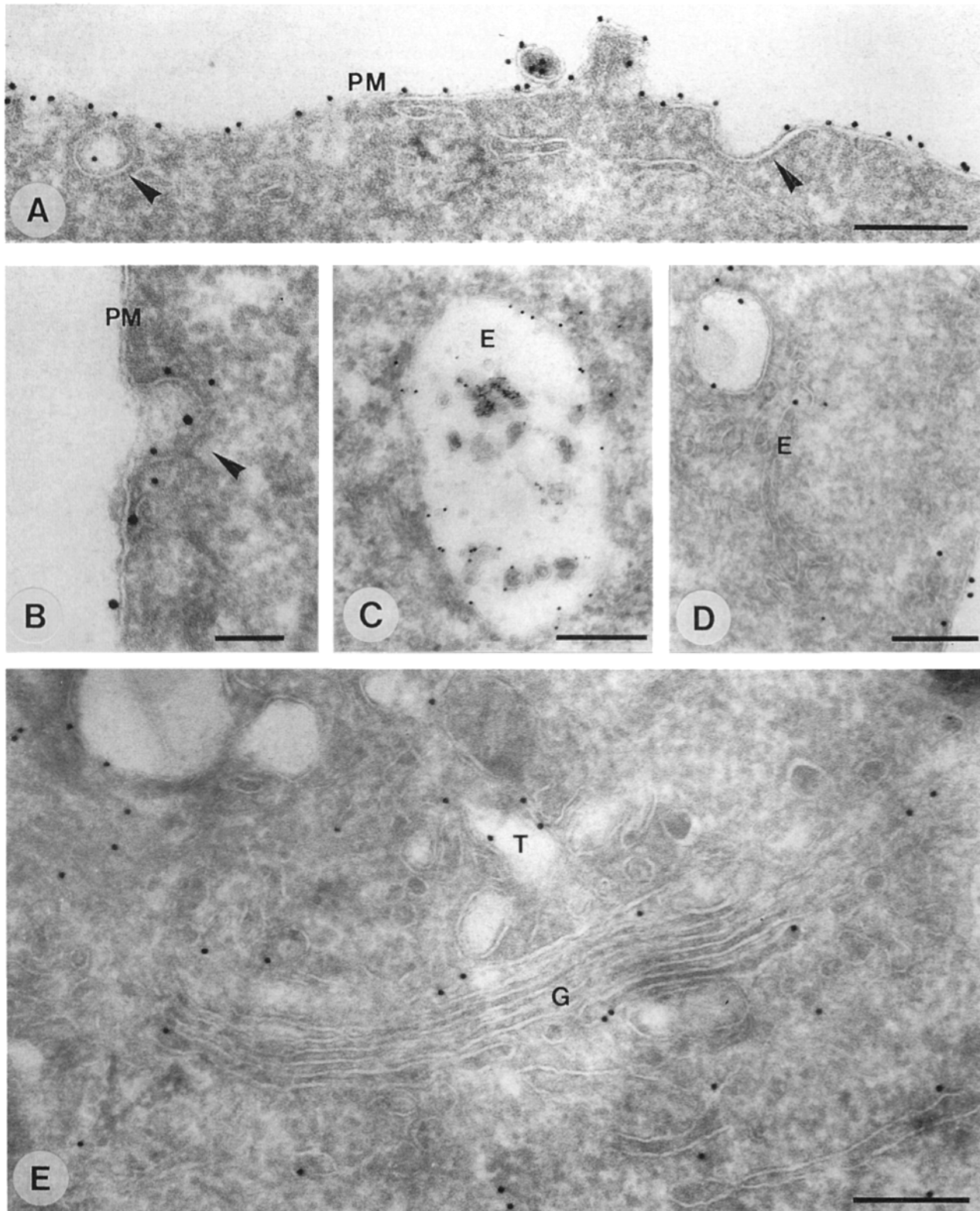


Figure 9. Immunolocalization of MFR- α in ultrathin cryosections of KB cells. Ultrathin cryosections of cultured KB cells which were incubated with BSA coupled to 5-nm gold particles for 1 h before fixation. The sections were immunolabeled with anti-(MFR- α) and protein A-coupled gold particles (Au). (A) Abundant and homogeneous labeling for MFR- α (10 nm Au) at the plasma membrane (PM) and in small invaginations of the plasma membrane, presumably clathrin-coated pits (arrowheads). (B) MFR- α (15 nm Au) colocalizes with clathrin (10 nm Au) in clathrin-coated pits (arrowheads) at the plasma membrane. (C and D) MFR- α (10 nm Au) was found in a variety of small and large, endocytic structures (containing BSA coupled to 5 nm Au). (E) Abundant labeling for MFR- α in the Golgi stack (G) and in the trans-Golgi network (T). Bars: (A) 200 nm; (B) 100 nm; (C) 200 nm; (D) 250 nm; (E) 200 nm.

Table I. Relative Distribution of MFR- α Immunoreactive Sites in KB Cells as Measured with Immunoelectron Microscopy on Ultrathin Cryosections

	Number of cells	Plasma membrane	Endocytic compartment	Biosynthetic compartment	Miscellaneous
Exp 1	10	88.18	9.00	1.87	0.99
Exp 2	10	84.75	11.30	2.49	1.46
Exp 3	10	90.25	7.59	1.43	0.73
Average		87.71	9.30	1.93	1.06
SEM		1.60	1.08	0.31	0.21

The relative distribution was determined by counting the gold particles found over each cell profile. From that the relative distribution was calculated and averaged over 10 cells.

Plasma membrane: Plasma membrane, coated pits, and caveolae.

Endocytic compartment: Small tubulo-vesicular structures, endosomes, multivesicular endosomes.

Biosynthetic compartment: Rough endoplasmic reticulum, Golgi complex, and *trans*-Golgi reticulum.

Miscellaneous vesicles: Vesicles which could not be assigned to one of the previously defined categories.

This is in good agreement with the study of Mayor et al. (1994), in which the authors showed that the clustering of MFR- α in monkey kidney MA104 cells was induced by the application of the anti-MFR antibody to the cell surface of unfixed cells. Therefore, the different localizations of MFR- α found by the different groups arise from differences in the methods used.

Triton X-100-insoluble membrane domains have been suggested to represent caveolae, which are enriched in GPI-linked proteins (Fiedler et al., 1993; Sargiacomo et al., 1993) including MFR- α (this study). Caveolae have been proposed to function in the uptake of folates in a process which has been termed potocytosis. In this process folates are concentrated by the clustering of folate receptors in caveolae, which close without pinching off and subsequently acidify causing the folates to dissociate from the receptors. The folates would then enter the cytosol by the action of an as yet unidentified folate transporter (Rothberg et al., 1990; Anderson, 1993a). The RFC has been shown to be capable of translocating folates across the membrane into the cytosol. In our experiments the RFC distributes on the bottom of the sucrose flotation gradients and is excluded from the detergent resistant membrane domains. This indicates that the RFC is not a likely candidate to act in tandem with MFR- α in caveolae to translocate folates into the cytosol. The RFC has been shown to be capable of translocating folates directly at the plasma membrane without an obligatory concentration of the vitamin by MFRs before translocation (Sirotnak, 1985; Jansen et al., 1990b), even under conditions where folate uptake via MFR- α was blocked (Westerhof et al., 1991, 1994). In addition, several cell lines have been identified which lack MFRs but express an active RFC, such as the CEM-7A cells used in this study. On the other hand, the fact that cells can survive by means of a GPI-linked folate receptor is illustrated by a previous study in which human leukemia cells with a defective RFC were shown to grow under nanomolar concentrations of folates (Jansen et al., 1989b). Therefore, it has been proposed that the RFC is the preferred folate-uptake system in cells that express both systems (Westerhof et al., 1993, 1994), although cells lacking the RFC can accumulate sufficient amounts of folates to grow by means of MFRs.

The fact that MFR- α has a GPI anchor opens the possibility for a role in signal transduction cascades, as has been suggested for other GPI-linked proteins such as Thy-1 and Decay Accelerating Factor (Stefanová et al., 1991; Shenoy

et al., 1992; Thomas and Samuelson, 1992; Dráberová and Dráber, 1993). GPI-anchored MFRs have also been implicated in signal transduction causing a proliferative response in hematopoietic progenitor cells (Antony et al., 1987, 1991) and an increased transient uptake of Ca²⁺ in *Dictyostelium Discoideum* (Milne and Coukell, 1991). Recently, it has been suggested that caveolae function as a specialized region of the plasma membrane for Ca²⁺-homeostasis and signal transduction (Anderson, 1993a,b; Lisanti et al., 1994b). In contrast to this hypothesis is the report by Fra et al. (1994) in which the authors show the presence of detergent-insoluble glycolipid domains in cells devoid of caveolae and caveolin.

In conclusion, we feel that the role of GPI-linked MFRs in the uptake of (anti-)folates is subordinate to the role of the RFC. This does not alter the fact that MFRs can be used to introduce antifolate compounds (ZD1694, CB3717, and DDATHF) with high affinity for MFRs into neoplastic cells, such as in ovarian cancer in which MFR- α has been suggested to be a marker protein because of its constant high level of expression (Campbell et al., 1991). The possible role of MFRs in signal transduction cascades involved in cell proliferation offers exciting new possibilities for investigation.

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