A Short Peptide Motif at the Carboxyl Terminus Is Required for Incorporation of the Integral Membrane MAL Protein to Glycolipid-enriched Membranes*

Rosa Puertollano‡ and Miguel A. Alonso§

From the Centro de Biología Molecular “Severo Ochoa,” Universidad Autónoma de Madrid, Consejo Superior de Investigaciones Científicas, Cantoblanco, 28049-Madrid, Spain

The MAL (VIP17, MVP17) proteolipid, an integral membrane protein with specific residence in glycolipid-enriched membrane (GEM) microdomains, has been recently proposed as a component of the protein machinery for GEM solubilization. In this work, we have searched the COOH terminus of MAL for sorting determinants responsible for targeting to GEMs. This has allowed the identification of the sequence Leu-Ile-Arg-Trp (LIRW) as necessary for the access of MAL to GEMs. This motif requires at least one additional amino acid at its COOH end for full effectiveness. The arginine within the LIRW motif is the most crucial residue for targeting to GEMs, tryptophan replacement affects targeting to a lesser extent, and the leucine-isoleucine pair tolerates substitution by valine, but not by alanine, without effect on targeting. Pulse-chase experiments indicate that the LIRW tetrapeptide is required for access to GEMs early after MAL biosynthesis. Interestingly, the loss of the capacity of the MAL protein to be incorporated into GEMs correlated with the loss of its response to brefeldin A treatment. This is the first identification of a juxtamembrane peptide motif required for incorporation of an integral membrane protein into GEMs.

Glycolipid-enriched membranes (GEMs)1 resistant to detergent solubilization have been characterized in many different cell types (1–4). Internal GEMs have been proposed to play a role in polarized transport of glycosylphosphatidylinositol-anchored proteins and glycolipids to the apical surface in Madin-Darby canine kidney (MDCK) cells (5, 6). According to this model, glycolipid self-association into GEM microdomains or “rafts” provides the biophysical basis for the recruiting of lipids and proteins. In addition, the existence of a specialized protein machinery to make the GEM rafts operative as a route of transport vesicles (7). Characterization of the proteins present in the GEM fraction from apical vesicles has revealed the identity of four of these proteins: caveolin (caveolin-1, VIP21) (8), initially identified as component of caveolar structure (9); VIP36 (10), a lectin homologous to leguminous lectins; annexin XIIIb (11), a member of the epithelial specific annexin XIII subfamily; and the MAL (VIP17) proteolipid protein (12). An interesting point is to know how the protein machinery is targeted to GEMs.

The human MAL gene was initially identified during a search for genes differentially expressed during T cell development (13). Despite its restricted pattern of expression (13, 14), the MAL protein has been identified in myelin-forming cells (4, 15) and in both polarized MDCK (12) and thyroid epithelial cells (16). The MAL gene encodes a nonglycosylated integral membrane protein of 17 kDa containing multiple hydrophobic segments (13). In contrast to most integral membrane proteins, MAL displays a high solubility in organic solvents used to extract cell lipids (17). This unusual lipid-like physical-chemical property allowed the isolation of MAL to the proteolipid group of proteins (18). In addition, MAL shares with a very restricted group of integral membrane proteins the distinctive biochemical feature of residence in GEMs (4, 12, 16, 19). This property, together with its distribution in vesicular structures (19), the co-isolation of MAL with the proteolipid group of proteins (18). In vesicles containing apically destined proteins in MDCK cells (12), and the distribution of endogenous MAL at the apical zone of polarized epithelial thyroid cells (16), are all consistent with a role for MAL as a protein component of the machinery for GEM-mediated apical transport. Ectopic expression of MAL in insect SF21 cells induces de novo formation of vesicular structures (20). Interestingly, the MAL-induced vesicles are different from the caveola-like vesicles induced by caveolin expression using the same expression system (20). These results, together with those showing MAL and caveolin in different GEM microdomains in MDCK cells (21), have been interpreted as that MAL and caveolin might be responsible for the generation of different vesicular carriers likely involved in distinct GEM-mediated pathways (20). These two pathways might work simultaneously in cells expressing both MAL and caveolin (MDCK cells), or separately in cells expressing either only MAL (T lymphocytes and oligodendrocytes) or caveolin (fibroblasts) (21).

To analyze whether the ability of MAL to access to GEMs relies on amino acid sorting determinants present in its carboxyl terminus, we have generated and studied the distribution of a panel of MAL mutants. This approach has allowed the identification of a LIRW tetrapeptide required for the specific incorporation of MAL into GEMs and for its localization in vesicles containing apically destined proteins in MDCK cells (12), and the distribution of endogenous MAL at the apical zone of polarized epithelial thyroid cells (16), are all consistent with a role for MAL as a protein component of the machinery for GEM-mediated apical transport. Ectopic expression of MAL in insect SF21 cells induces de novo formation of vesicular structures (20). Interestingly, the MAL-induced vesicles are different from the caveola-like vesicles induced by caveolin expression using the same expression system (20). These results, together with those showing MAL and caveolin in different GEM microdomains in MDCK cells (21), have been interpreted as that MAL and caveolin might be responsible for the generation of different vesicular carriers likely involved in distinct GEM-mediated pathways (20). These two pathways might work simultaneously in cells expressing both MAL and caveolin (MDCK cells), or separately in cells expressing either only MAL (T lymphocytes and oligodendrocytes) or caveolin (fibroblasts) (21).

To analyze whether the ability of MAL to access to GEMs relies on amino acid sorting determinants present in its carboxyl terminus, we have generated and studied the distribution of a panel of MAL mutants. This approach has allowed the identification of a LIRW tetrapeptide required for the specific incorporation of MAL into GEMs and for its localization in vesicles containing apically destined proteins in MDCK cells (21), and the distribution of endogenous MAL at the apical zone of polarized epithelial thyroid cells (16), are all consistent with a role for MAL as a protein component of the machinery for GEM-mediated apical transport. Ectopic expression of MAL in insect SF21 cells induces de novo formation of vesicular structures (20). Interestingly, the MAL-induced vesicles are different from the caveola-like vesicles induced by caveolin expression using the same expression system (20). These results, together with those showing MAL and caveolin in different GEM microdomains in MDCK cells (21), have been interpreted as that MAL and caveolin might be responsible for the generation of different vesicular carriers likely involved in distinct GEM-mediated pathways (20). These two pathways might work simultaneously in cells expressing both MAL and caveolin (MDCK cells), or separately in cells expressing either only MAL (T lymphocytes and oligodendrocytes) or caveolin (fibroblasts) (21).

To analyze whether the ability of MAL to access to GEMs relies on amino acid sorting determinants present in its carboxyl terminus, we have generated and studied the distribution of a panel of MAL mutants. This approach has allowed the identification of a LIRW tetrapeptide required for the specific incorporation of MAL into GEMs and for its localization in vesicles containing apically destined proteins in MDCK cells (21), and the distribution of endogenous MAL at the apical zone of polarized epithelial thyroid cells (16), are all consistent with a role for MAL as a protein component of the machinery for GEM-mediated apical transport. Ectopic expression of MAL in insect SF21 cells induces de novo formation of vesicular structures (20). Interestingly, the MAL-induced vesicles are different from the caveola-like vesicles induced by caveolin expression using the same expression system (20). These results, together with those showing MAL and caveolin in different GEM microdomains in MDCK cells (21), have been interpreted as that MAL and caveolin might be responsible for the generation of different vesicular carriers likely involved in distinct GEM-mediated pathways (20). These two pathways might work simultaneously in cells expressing both MAL and caveolin (MDCK cells), or separately in cells expressing either only MAL (T lymphocytes and oligodendrocytes) or caveolin (fibroblasts) (21).

To analyze whether the ability of MAL to access to GEMs relies on amino acid sorting determinants present in its carboxyl terminus, we have generated and studied the distribution of a panel of MAL mutants. This approach has allowed the identification of a LIRW tetrapeptide required for the specific incorporation of MAL into GEMs and for its localization in vesicles containing apically destined proteins in MDCK cells (21), and the distribution of endogenous MAL at the apical zone of polarized epithelial thyroid cells (16), are all consistent with a role for MAL as a protein component of the machinery for GEM-mediated apical transport. Ectopic expression of MAL in insect SF21 cells induces de novo formation of vesicular structures (20). Interestingly, the MAL-induced vesicles are different from the caveola-like vesicles induced by caveolin expression using the same expression system (20). These results, together with those showing MAL and caveolin in different GEM microdomains in MDCK cells (21), have been interpreted as that MAL and caveolin might be responsible for the generation of different vesicular carriers likely involved in distinct GEM-mediated pathways (20). These two pathways might work simultaneously in cells expressing both MAL and caveolin (MDCK cells), or separately in cells expressing either only MAL (T lymphocytes and oligodendrocytes) or caveolin (fibroblasts) (21).

To analyze whether the ability of MAL to access to GEMs relies on amino acid sorting determinants present in its carboxyl terminus, we have generated and studied the distribution of a panel of MAL mutants. This approach has allowed the identification of a LIRW tetrapeptide required for the specific incorporation of MAL into GEMs and for its localization in vesicles containing apically destined proteins in MDCK cells (21), and the distribution of endogenous MAL at the apical zone of polarized epithelial thyroid cells (16), are all consistent with a role for MAL as a protein component of the machinery for GEM-mediated apical transport. Ectopic expression of MAL in insect SF21 cells induces de novo formation of vesicular structures (20). Interestingly, the MAL-induced vesicles are different from the caveola-like vesicles induced by caveolin expression using the same expression system (20). These results, together with those showing MAL and caveolin in different GEM microdomains in MDCK cells (21), have been interpreted as that MAL and caveolin might be responsible for the generation of different vesicular carriers likely involved in distinct GEM-mediated pathways (20). These two pathways might work simultaneously in cells expressing both MAL and caveolin (MDCK cells), or separately in cells expressing either only MAL (T lymphocytes and oligodendrocytes) or caveolin (fibroblasts) (21).
peculiar physical-chemical properties of the MAL molecule. This is the first report showing the requirement for a juxtapamembrane peptide motif in the sorting of an integral membrane protein to GEMs.

**EXPERIMENTAL PROCEDURES**

**Materials**—The mouse hybridomas producing monoclonal antibody (mAb) to the 9E10 c-Myc epitope (EQLKLISEED) (22) or to human CD4 were purchased from the American Type Culture Collection. Rabbit polyclonal antibodies to transferrin were from Dako A/S (Glostrup, Denmark). BPA, nocodazole, Triton X-100, and iron-saturated human transferrin were from Sigma. Peroxidase-conjugated rabbit anti-mouse IgG antibodies were from Pierce. Fluorescein- and Texas Red-conjugated antibodies were from Southern Biotech (Birmingham, AL).

**DNA Constructions**—The insertion of the 9E10 c-Myc epitope between the first and the second amino acid of MAL was carried out by amplification of the AMA5 insert (13) with a polymerase chain reaction using the oligonucleotide primers N (5'-GGG CCC AGA TCT CAT ATG GAG CAG ATC CTG TCC GAG GAA CAC GTG CCC GCC GCA GCG GCG ACG-3') and C (5'-CCC GGG AGA TCT TTA TTA TGA AGT CTT CCA TCT-3') (Isogen Bioscience, Amsterdam, The Netherlands). These primers contain sequences which anneal to the 5' ends of the CD4 coding sequences and CD4 cDNA (26) and contain one BglII site at the 5' end. After amplification under standard conditions (23), the product was digested with BglII and cloned into the unique BamHI site of the pSRα expression vector (24). Mutations at the COOH terminus of MAL by deletion, addition, or substitution of amino acids were performed in a similar way by the polymerase chain reaction using primer N and, in each case, a specific oligonucleotide primer carrying both the appropriate modifications and a sequence complementary to the 3' end of the MAL coding sequence. After digestion of a BglII target sequence present in each oligonucleotide primer, the amplification product was cloned into pSRα. The cDNA corresponding to the 16-kDa proteolipid of the bovine V-ATPase (25) was tagged at the NH2 terminus with a sequence encoding the 9E10 c-Myc epitope by amplification of the corresponding cDNA (a kind gift from Dr. N. Nelson, Roche Institute of Molecular Biology, Nutley, NJ) with appropriate primers. For DNA constructions expressing CD4 we used primers specific for the 5' and 3' ends of the CD4 coding sequences and CD4 cDNA (26) as template (a kind gift from Dr. P. J. Maddon, Columbia University, New York). The constructs expressing chimeric proteins modified at their carboxyl terminus with MAL sequences were done by amplification with appropriate oligonucleotide primer pairs. The sequence of the inserted protein was verified in all of the constructs to eliminate the possibility of amplification errors.

**Cell Culture Conditions and Transfection**—Human epithelial A498 cells (ATCC HB44) and COS-7 cells were grown on Petri dishes or glass coverslips, in Dulbecco's modified Eagle's medium supplemented with 1% of fetal bovine serum (Life Technologies, Inc.), penicillin (100 units/ml), and 100 μg/ml streptomycin. The mouse hybridomas producing monoclonal antibody (mAb) to the 9E10 c-Myc epitope were maintained in drug-free medium. More than 90% of the cells were finally brought to 40% sucrose in a final volume of 4 ml and placed in a 4°C nitrogen atmosphere. After this period, the medium was removed and replaced with standard culture medium. To be used in immunoprecipitation studies, antibodies were prebound overnight at 4°C to protein G-Sepharose in 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% Triton X-100. Cell extracts were incubated for 4 h at 4°C with an irrelevant control antibody bound to protein G-Sepharose and centrifuged, and the supernatant was immunoprecipitated by incubation for 4 h at 4°C with the appropriate antibodies bound to protein G-Sepharose. Immunoprecipitates were washed six times with 1 ml of 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% Triton X-100, and analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. To detect [35S]labeling, dried gels were finally exposed to Fuji film imaging plates.

**Immunofluorescence Microscopy**—Epithelial A498 cells grown on coverslips were washed twice with PBS, fixed in 3% formaldehyde for 15 min, rinsed, and treated with 10 mM glycine for 10 min to quench the aldehyde groups. The cells were then permeabilized with 0.2% Triton X-100, rinsed, and incubated with 3% bovine serum albumin in PBS for 20 min. Coverslips were then incubated with the primary antibody 1 h, rinsed several times, and incubated for one additional hour with the appropriate fluorescent secondary antibody. For double-label immunofluorescence analysis the procedure was repeated again with the second primary and secondary antibodies. After extensive washing, the coverslips were mounted on slides. The cells were photographed with a Zeiss Axiopt2 photomicroscope using Kodak T-Max 400 film. Primary antibodies included mouse mAb 9E10 (IgG1), used as a culture supernatant, and rabbit polyclonal antibodies to transferrin used at 1:10,000 dilution. Secondary antibodies included Texas Red-conjugated goat anti-mouse IgG, antibodies used at 1:1,000, and fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies absorbed against mouse IgG used at 1:100. Controls to assess the specificity and the lack of cross-labeling included incubations with an irrelevant primary mAb or omission of either of the primary antibodies.

**RESULTS**

**Deletion of the Last Four Amino Acids from the COOH Terminus Prevents Targeting of MAL to GEMs**—The last nine amino acids (FSLRKWSS) from the COOH terminus of the MAL protein contain sequences that resemble distinct signals involved in protein sorting. Thus, the FSLI sequence is reminiscent of tyrosine-based amino acid motifs involved in endocytosis or basolateral sorting (29, 30). The LI pair might correspond to LI-based signals that act in endo-sorting or basolateral sorting (31, 32). The sequence FSLIRW fits the difference X(X)/X-aromatic sequence pattern involved in protein internalization (33). Finally, the RKWSS sequence resembles both COOH-terminal double-lysine KRXKXXX signals involved in the retrieval of type I transmembrane proteins to the endoplasmic reticulum (34, 35) and KXXX motifs involved in endocytosis (36). To explore whether any of these four motifs or other novel sequence in the same region is involved in the
Targeting of MAL Proteolipid to Glycolipid-enriched Membranes

Fig. 1. Representative immunoblots of the subcellular fractionation profiles obtained by centrifugation to equilibrium in sucrose density gradients. A498 cells stably expressing the MAL protein tagged with a c-Myc epitope at its NH2 terminus, or variants of this protein with mutations at the COOH-terminal end were extracted at 4 °C with 1% Triton X-100 and subjected to centrifugation to equilibrium in sucrose density gradients following standard procedures (27). After fractionation from the bottom of the tube, aliquots from the different fractions were subjected to immunoblot analysis with anti-c-Myc mAb 9E10. Fractions 1–4 are the 40% sucrose layer and contain the bulk of cellular membranes and cytosolic proteins, while fractions 5–12 are the 5–30% sucrose layer and contain GEMs (3, 27). Fractions 1–4 include >99% of total cellular proteins as shown previously (3). Fraction 5, which is close to the 30–40% sucrose interphase, appeared occasionally contaminated with soluble proteins as revealed by Coomasie Blue staining of the blot. In those cases, fraction 5 was considered as belonging to the soluble fractions. For simplicity, the different mutants assayed were included in one of the four indicated categories: (+ + + +), (+ + +), (+ +) and (−) corresponding to 95–100, 90–95, 50–90, or ≤5% of the MAL protein in GEM fractions, respectively. The profiles shown correspond to MAL (+ + + +), MAL-FSLIRWKSS (+ + +), MAL-FSLSWKSS (+), and MAL-FSLIR (−).

Identification of a Peptide Motif at the MAL COOH Terminus Required for Targeting to GEMs—The results with the deletional mutants discussed above indicated that the last two serines in the MAL molecule are dispensable for the incorporation of MAL into GEMs. Whereas the deletion of the last two amino acids (MAL-FSLIRWK) did not affect the targeting of MAL, truncation of three (MAL-FSLIRW) or four (MAL-FSLSIR) carboxy-terminal amino acids progressively diminished the level of MAL in GEMs. Elongation of the COOH-terminal end with three additional serines (MAL-FSLIRWKSSSSSS) did not affect the efficiency of the targeting, in agreement with our previous results showing that the addition of a tag to the carboxyl terminus did not show any effect in the incorporation of MAL into GEMs (20).

### Table I

| Mutant Incorporation into GEMs | Group I | Group II | Group III | Group IV |
|-------------------------------|---------|----------|-----------|----------|
| MAL-FSLIRWKSS                 | ++      | ++       | +         | -        |
| MAL-FSLIRWK                  | ++      | ++       | ++        | -        |
| MAL-FSLIR                   | ++      | ++       | ++        | -        |
| MAL-FSLIRWSSSS               | +       | ++       | ++        | -        |
| MAL-FSLIRWK                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |
| MAL-FSLSIR                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |

Specific targeting of MAL to GEMs, we have generated a panel of deletional, insertional, and replacement mutants at the COOH terminus of MAL. In all of the cases, the MAL protein was tagged at its NH2 terminus with the 9E10 c-Myc epitope (22). Since this tag does not interfere the targeting of MAL to GEMs (16, 21), from now on we will refer the tagged MAL protein lacking any further modification as MAL. As all the mutations were concentrated in the last nine amino acids, mutants were named by the amino acid sequence in that region. The different MAL proteins were stably expressed in transfected human epithelial A498 cells selected by prolonged treatment with the aminoglycoside G418. Triton X-100 extracts from clones of the different transfectants were subjected to centrifugation to equilibrium in sucrose density gradients following an established protocol (27). After fractionation from the bottom of the tube, aliquots from the different fractions were analyzed by immunoblotting with anti-c-Myc mAb 9E10. Using this protocol, fractions 1–4 correspond to cytosolic proteins and solubilized membranes, whereas fractions 5–12 consist of the buoyant material containing GEMs resistant to detergent solubilization. As a control we first checked that GEMs containing the same pattern of endogenous cellular proteins were obtained from both untransfected and transfected cells, indicating that GEMs preexist in the cell and they are neither formed nor destroyed by expression of the different MAL proteins (not shown). For simplicity, the different profiles obtained were classified into four distinct categories: (+ + + +), (+ + +), (+ +) and (−) corresponding to ≥90, 90–20, 20–5, or ≤5% of the MAL protein incorporated into GEM fractions, respectively. In none of the mutants, the incorporation of MAL into GEMs was between 50 and 90%. Representative profiles for the four categories are presented in Fig. 1.

The first group of mutants analyzed contained alterations in the length of the COOH-terminal tail of MAL by either amino acid deletion or addition (Table I, group I). In agreement with previous reports (19), MAL completely distributed to the fractions containing GEMs. Whereas the deletion of the last two amino acids (MAL-FSLIRWK) did not affect the targeting of MAL, truncation of three (MAL-FSLIRW) or four (MAL-FSLSIR) carboxy-terminal amino acids progressively diminished the level of MAL in GEMs. Elongation of the COOH-terminal end with three additional serines (MAL-FSLIRWKSSSSSS) did not affect the efficiency of the targeting, in agreement with our previous results showing that the addition of a tag to the carboxyl terminus did not show any effect in the incorporation of MAL into GEMs (20).

### Table I

| Mutant Incorporation into GEMs | Group I | Group II | Group III | Group IV |
|-------------------------------|---------|----------|-----------|----------|
| MAL-FSLIRWKSS                 | ++      | ++       | ++        | -        |
| MAL-FSLIRWK                  | ++      | ++       | ++        | -        |
| MAL-FSLIR                   | ++      | ++       | ++        | -        |
| MAL-FSLIRWSSSS               | +       | ++       | ++        | -        |
| MAL-FSLIRWK                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |
| MAL-FSLSIR                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |

The second group of mutants contained deletions of tryptophan residues at the MAL COOH terminus (Table I, group II). In agreement with previous reports (22), the deletion of the last two tryptophans (MAL-FSLSIR) caused a significative drop on the level of MAL in the COOH terminus of MAL. To address whether the tryptophan residues are themselves forming part of a motif required for MAL residency in GEMs or just playing a role as spacers to place an unidentified distance-dependent signal in optimal position, we analyzed MAL mutants with substitutions at the COOH terminus (Table I, group II). Substitution of the last two tryptophans (MAL-FSLSIRWSSSS) did not affect the presence of MAL in GEMs, suggesting that the tryptophan residues are not required for MAL targeting. The replacement of tryptophan by serine (MAL-FSLSIRSSKSS) caused a significative drop on the level of MAL in the

| Mutant Incorporation into GEMs | Group I | Group II | Group III | Group IV |
|-------------------------------|---------|----------|-----------|----------|
| MAL-FSLIRWKSS                 | ++      | ++       | ++        | -        |
| MAL-FSLIRWK                  | ++      | ++       | ++        | -        |
| MAL-FSLIR                   | ++      | ++       | ++        | -        |
| MAL-FSLIRWSSSS               | +       | ++       | ++        | -        |
| MAL-FSLIRWK                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |
| MAL-FSLSIR                  | ++      | ++       | ++        | -        |
| MAL-FSLSIRW                  | ++      | ++       | ++        | -        |
Targeting of MAL Proteolipid to Glycolipid-enriched Membranes

Fig. 2. Immunofluorescence analysis of MAL mutants unable to access to GEMs. The steady-state distribution in A498 cells of MAL mutants incorporated ≤5% into the GEM fractions as well as their response to treatment with either nocodazole or BFA were compared with similar analyses in cells expressing MAL. As all of the mutants unable to be incorporated into GEMs gave identical results only the experiments with MAL-FSLIR are shown. Early endosomes were pre-loaded by incubation of the cells with 0.5 mg/ml transferrin for 1 h. The effect of BFA on transferrin was used as an internal control for the BFA effect. The distribution of the indicated MAL proteins in untreated cells (A and E) or in cells treated with 20 μg nocodazole for 30 min (B and F) was analyzed by immunofluorescence analysis. The distribution of transferrin (D and H) in cells treated with 5 μg/ml BFA for 30 min analyzed by double-label immunofluorescence analysis. Bar, 3 μm.

GEM fractions. Finally, substitution of arginine –5 by serine (MAL-FSLISWKSS) greatly affected the targeting of MAL to GEMs.

The third group of mutants were generated to analyze the influence of the FSLL- and LI dipeptides on the targeting of MAL (Table I, group III). In this panel of mutants the last two serines were deleted as they were dispensable for the access of MAL to GEMs. Whereas en bloc substitution of FSLL by alanine (MAL-AAAAARWKK) impaired the ability of MAL to reside in GEMs, the replacement of FS by two alanines (MAL-AA-LIRWK) did not show any effect. Consistently, replacement of the LI dipeptide by alanine (MAL-FSAAARWKK) greatly diminished the presence of MAL in the GEM fractions. Individual substitutions in the LI dipeptide by alanine (MAL-FSAAIRWKK and MAL-FSLARWKK) showed that both leucine and isoleucine were similarly required. The LI pair was substituted by valine (MAL-FSVVRWK) without affecting the targeting of MAL to GEMs. Finally, MAL-FSVVR presented both the same solubility and distribution as that of MAL-FSLIR (Fig. 2E).

The MAL mutants described so far delineated a COOH-terminal LIIRW tetrapeptide followed by at least one additional amino acid as a motif required for the targeting of MAL to GEMs. The LI pair in this sequence can be substituted by a bulky hydrophobic amino acid such as valine but not by a small residue such as alanine without affecting the efficiency of targeting. To further analyze the requirement for the RW pair, we generated a fourth group of mutants (Table I, group IV) in which arginine was substituted by lysine (MAL-FSLIKWK), or tryptophan replaced by either alanine (MAL-FSLIRAK), leucine (MAL-FSLIRLK) or phenylalanine (MAL-FSLIRFK).

None of these mutations was able to fully replace the RW pair in its capacity to target MAL to GEMs. Finally, when the LI pair was separated from the RWKSS sequence by insertion of three serine residues (MAL-FSLISSRSSWKSS), this mutant was excluded from GEMs.

The Failure of MAL to Be Incorporated into GEMs Correlates with Unresponsiveness of MAL to BFA Treatment.—Immunofluorescence analysis in A498 cells expressing MAL revealed that MAL is concentrated at steady state in vesicular structures in the perinuclear region with little peripheral labeling (Fig. 2A). As all of the mutants that failed to incorporate into GEMs showed the same distribution, only the result with MAL-FSLIR is shown (Fig. 2E). MAL-FSLIR accumulated into the perinuclear region in vesicular structures that were larger than those containing MAL. BFA treatment of cultured cells causes the Golgi to redistribute to the endoplasmic reticulum and the trans-Golgi network to fuse with early endosomes forming an extensive trans-Golgi network/early endosomes tubular network (37). In order to compare the BFA sensitivity of the wild type protein and that of MAL-FSLIR, we carried out immunofluorescence analysis in stably transfected A498 cells. The cells were pre-loaded with transferrin to label early endosomes (38) and treated with BFA for 30 min. The formation of tubules containing transferrin was used as an internal control for the efficiency of the treatment. After incubation in the presence of BFA, MAL was found in a discontinuous array of vesicles that were distributed along tubules stained in a continuous pattern by anti-transferrin antibodies (Figs. 2, C and D). Although BFA affects the distribution of the vesicles containing MAL, previous work demonstrated that BFA does not have any effect on the residence of MAL in GEMs (19). In contrast to MAL, MAL-FSLIR does not tubulate even after long periods in the presence of BFA. The distribution of transferrin in the same cells after BFA treatment is shown as a control of the effect of BFA (Fig. 2H). Nocodazole is a microtubule disrupting agent known to cause the disassembly of cellular organelles requiring microtubule integrity (39). Fig. 2, B and F, shows that the vesicles containing MAL or MAL-FSLIR, respectively, were completely distributed to the cell periphery after treatment for 30 min in the presence of nocodazole.

The LIIRW Motif Is Not Sufficient to Target Other Integral Membrane Proteins to GEMs—To investigate whether the LIIRW motif is able to target other integral membrane proteins to GEMs, we made chimeras with the CD4 glycoprotein, chosen as a representative of type I integral membrane proteins, and with the 16-kDa subunit of the vacuolar H⁺-ATPase, selected because it shares with MAL a similar size and hydrophobicity profile (17). The CD4 chimeras were constructed by appending the MAL carboxyl-terminal sequences containing the LIIRW motif either to the COOH-terminal tail of CD4 or after its transmembrane domain. In the case of the 16-kDa subunit of the vacuolar H⁺-ATPase, the LIIRW sequence was appended to its carboxyl terminus. Neither of the chimeras assayed were targeted to GEMs when assayed in transient expression experiments in COS cells (not shown). This suggests that, although the LIIRW motif is able to target MAL to GEMs, either other determinants or global physical-chemical properties of MAL may be also necessary for access to GEMs.

Mutations at the LIIRW Motif That Affect MAL Targeting Do Not Influence MAL Oligomerization.—The formation of large oligomers is one of the targeting mechanisms used by integral membrane proteins for incorporation into the appropriate membranes (40). To address whether modification in the oligomerization of the MAL molecule in the mutants with an
altered LIRW motif was responsible for exclusion from GEMs, we analyzed the oligomeric state of MAL-FSLIRWK and MAL-FSLIKWK by velocity gradient centrifugation. These two mutants were chosen as representatives of the groups of proteins able or unable to get targeted to GEMs, respectively. Fig. 3 shows that the sedimentation profile was similar for the two proteins, indicating that substitution of arginine by lysine within the LIRW motif does not affect MAL oligomerization.

The LIRW Motif Is Required for MAL Access to GEMs—The results shown so far indicate that the LIRW motif found at the COOH terminus of MAL is required for residence of MAL in GEMs. Since in all those experiments MAL was analyzed at steady state, it remains to be established whether the LIRW motif sorts MAL to GEMs or promotes its retention in these membranes. To address this point we carried out pulse-chase experiments in stably transfected A498 cells. Cells stably expressing either MAL-FSLIRWK, which was shown to be correctly targeted, or MAL-FSLIKWK, which was totally mistargeted, were metabolically labeled for 10 min with [35S]methionine/cysteine and incubated in chase medium containing unlabeled amino acids for the indicated times. After extraction in 1% Triton X-100 at 4 °C, the lysates were adjusted to 40% sucrose and overlaid with a linear 5–30% sucrose density gradient. After centrifugation to equilibrium, separated pools of fractions 1–4 (S, soluble) and GEM-containing fractions 7–9 (I, insoluble) were adjusted to the same volume and buffer conditions and subjected to immunoprecipitation with anti-c-CD4 mAb, used as an irrelevant control antibody, or with anti-c-Myc mAb 9E10.

A LIRW Motif at the COOH Terminus Is Required for Both Specific Access of MAL to GEMs and MAL Sensitivity to BFA—As a result of our mutational analysis at the COOH-terminal tail, we have identified the sequence LIRW as necessary for the specific targeting of MAL to GEMs. This tetrapeptide signal requires at least one additional amino acid at its COOH terminus for optimal function. Interestingly, the MAL proteins harboring mutations that block incorporation of MAL into GEMs were localized in vesicular structures that, opposite to the case of the wild type protein, were insensitive to BFA. This suggests that the LIRW motif is necessary for both incorporation into GEMs and localization in BFA-sensitive vesicles. The single replacement of arginine in MAL-FSLIRWK by lysine makes MAL-FSLIKWK to be diverted from MAL-FSLIRWK during their biosynthetic transport. Whereas MAL-FSLIRWK is incorporated into GEMs early after biosynthesis, MAL-FSLIKWK was totally excluded. This indicates that the LIRW motif is required for the process of incorporation of MAL into GEMs. This finding strongly argues against that MAL insolubility is only due to the global physical-chemical properties of the protein or an artifact of the detergent extraction procedure. Although we have not observed any role in targeting to GEMs for the sequences resembling distinct consensus sorting signals present at the MAL carboxyl terminus, the possibility remains that these motifs might play a role in the modulation of MAL trafficking once MAL is incorporated into GEMs.

The inspection for the presence of the LIRW sequence at the COOH-terminal tail of proteins found in GEMs gave negative results. A sequence reminiscent of the MAL FSLIRW peptide is present in amino acids −7 to −12 of human (FSNVRI), mouse (FSNRI), chicken (FSSIRA), and dog (FSNIKI) caveolin (41). Although an exact LIRW sequence does not exist in caveolin, the presence in this molecule of an arginine residue in a context resembling to that in the MAL FSLIRW sequence led us to assay the effect of the deletion of the last twelve carboxyl-terminal amino acids on the targeting of dog caveolin to GEMs. No differences were found in the targeting of the mutant and the wild type protein (not shown), indicating that the deleted region is not necessary for the access of caveolin to GEMs. The fact that caveolin and MAL have different requirements for incorporation into GEMs is consistent with the presence of these proteins in distinct GEM microdomains (21). The investigation on the possible use of LIRW-related motifs in other proteins should await the identification of novel integral membrane proteins with residence in GEMs.

LIRW-dependent Sorting to GEMs Requires Additional Tar-
Targeting of MAL Proteolipid to Glycolipid-enriched Membranes

4. Kim, T., Fiedler, K., Madison, D. L., Krueger, W. H., and Pfeiffer, S. E. (1995) J. Neurosci. Res. 42, 413–422
5. Simons, K. and van Meer, G. (1988) Biochemistry 27, 6197–6202
6. Simons, K. and Wandinger-Ness, A. (1990) Cell 62, 207–210
7. Wandinger-Ness, A., Bennett, M. K., Antony, C., and Simons, K. (1990) J. Cell Biol. 111, 987–1000
8. Kurzchalia, T. V., Dupree, P., Parton, R. G., Kellner, R., Virta, H., Lehnert, M., and Simons, K. (1992) J. Cell Biol. 118, 1003–1014
9. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y., Glenney, J. R., and Anderson, R. G. W. (1992) Cell 68, 673–682
10. Fiedler, K., Parton, R. G., Kellner, R., Etxold, T., and Simons, K. (1994) EMBO J. 13, 1729–1740
11. Fiedler, K., Lafont, F., Parton, R. G., and Simons, K. (1995) J. Cell Biol. 128, 1083–1093
12. Zacchetti, D., Peranen, J., Murata, M., Fiedler, K., and Simons, K. (1995) FEBS Lett. 377, 465–469
13. Alonso, M. A. and Weissman, S. M. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 1997–2001
14. Tugores, A., Rubio, T., Rana%C3%A7o, C., and Alonso, M. A. (1997) DNA Cell Biol. 16, 245–255
15. Scharen-Wiemers, N., Valenzuela, D. M., Frank, M., and Schwab, M. E. (1995) J. Neurosci. 15, 5753–5764
16. Martin-Belmonte, F., Kremer, L., Albar, J. P., Marazuela, M., and Alonso, M. A. (1998) Endocytosis 139, 2077–2084
17. Rana%C3%A7o, C., Rubio, T., Correas, I., and Alonso, M. A. (1994) J. Biol. Chem. 269, 8159–8164
18. Weskämper, M. J. (1981) Annu. Rev. Biochem. 50, 193–206
19. Milian, J., Puertollano, R., Fan, L., Rana%C3%A7o, C., and Alonso, M. A. (1997) Biochem. J. 321, 247–252
20. Puertollano, R., Li, S., Lisanti, M. P., and Alonso, M. A. (1997) J. Biol. Chem. 272, 18311–18315
21. Milian, J., Puertollano, R., Fan, L., Alonso, M. A. (1997) Biochem. Biophys. Res. Commun. 233, 707–712
22. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Takebe, Y., Seiki, M., Fujisawa, J. H., Yokoy, K., Arai, K., Yoshida, M., and Arai, N. (1988) Mol. Cell. Biol. 8, 466–472
25. Mandle, M., Moriyama, Y., Hulmes, J. D., Pan, Y.-C. E., Nelson, H., and Nelson, N. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 5521–5524
26. Madden, P. J., Littman, D. R., Godfrey, M., Madden, D. E., Chess, L., and Axel, R. (1985) Cell 42, 93–104
27. Brown, D. A., and Rose, J. (1992) Cell 68, 533–544
28. Sargiacomo, M., Scherer, P. E., Tang, Z.-L., Kubiiek, E., Song, K. S., Sanders, M. C., and Lisanti, M. P. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 9407–9411
29. Sandovalo, I. V., and Bakke, O. (1994) Trends Cell Biol. 4, 292–297
30. Marks, M. S., Ohno, H., Kirchhausen, T., and Bonifacino, J. (1997) Trends Cell Biol. 7, 124–128
31. Sandovalo, I. V., Arredondo, J. J., Alcalde, J., Gonzalez Noriega, A., Vandenberghove, J., Jimenez, M. A., and Rico, M. (1994) J. Biol. Chem. 269, 6622–6631
32. Letourneur, F., and Klausner, R. D. (1992) Cell 69, 1143–1157
33. Towbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1995) Annu. Rev. Cell Biol. 9, 129–161
34. Jackson, M. R., Nilsson, T., and Peterson, P. A. (1990) EMBO J. 9, 3153–3162
35. Stob, J., Dunbrack, R. L., and Stroumpler, J. L. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 1918–1922
36. Itin, C., Kappeler, F., Linstedt, A. D., and Hauri, H.-P. (1995) EMBO J. 14, 2250–2256
37. Klausner, R. D., Donalson, J. G., and Lippincott-Schwartz, J. (1992) J. Cell Biol. 116, 1071–1080
38. Hopkins, C. R. (1983) Cell 35, 321–330
39. De Brabander, M. J., Von de Veire, R. M. L., Aerts, R. M. L., Borgers, M., and Janssen, P. N. J. (1976) Cancer Res. 36, 905–916
40. Weiss, O. A., Swift, A. M., and Machamer, C. A. (1993) J. Cell Biol. 122, 1135–1196
41. Tang, Z.-L., Scherer, P. E., Okamoto, T., Song, K., Chu, C., Kohtz, D. S., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 2255–2261
42. Matter, K., and Mellman, I. (1994) Curr. Opin. Cell Biol. 6, 545–554
43. Machamer, C. C. (1993) Curr. Opin. Cell Biol. 5, 606–612
44. Bretcher, M. S., and Munro, S. (1993) Science 261, 1280–12811
45. Nilsson, T., and Warren, G. (1994) Curr. Opin. Cell Biol. 6, 517–521
46. Munro, S. (1995) EMBO J. 14, 4695–4704
47. Nilsson, T., Hor, M. H., Slusarewicz, P., Rabouille, C., Watson, R., Hnte, F., Wistatiele, G., Berger, E. G., and Warren, G. (1994) EMBO J. 13, 562–574
48. Reithmeier, R. A. F. (1995) Curr. Opin. Struct. Biol. 5, 491–500
49. Deber, C., and Goto, N. K. (1996) Nat. Struct. Biol. 3, 815–818
50. Brown, D. (1993) Curr. Opin. Immunol. 5, 349–354
51. Brown, D. A., Crise, R., and Brown, J. K. (1989) Science 245, 1499–1501
52. Lisanti, M. P., Caras, I. W., Daito, M. A., and Rodriguez-Boulan, E. (1989) J. Cell Biol. 109, 2145–2156
53. Rodgers, W., Crise, R., and Rose, J. K. (1994) Mol. Cell. Biol. 14, 5384–5391
54. Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C., and Lublin, D. M. (1997) J. Cell Biol. 126, 353–363
55. Dietzen, D. J., Hastings, W. R., and Lublin, D. M. (1995) J. Biol. Chem. 270, 2680–2682
56. Song, K. S., Tang, Z.-L., Li, S., and Lisanti, M. P. (1997) J. Biol. Chem. 272, 4398–4403
57. Scheiffele, P., Roth, M. G., and Simons, K. (1997) EMBO J. 16, 5501–5508