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Substitution of the Two Carboxyl-terminal Serines by Alanine Causes Retention of MAL, a Component of the Apical Sorting Machinery, in the Endoplasmic Reticulum

Rosa Puertollano and Miguel A. Alonso
Centro de Biología Molecular "Severo Ochoa," Universidad Autónoma de Madrid, Consejo Superior de Investigaciones Científicas, Cantoblanco, 28049-Madrid, Spain

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MAL, a selective resident of glycolipid-enriched membranes (GEMs), is an integral membrane protein necessary for apical transport and accurate sorting of the influenza virus hemagglutinin in MDCK cells. The carboxyl-terminal end of MAL has the sequence Phe-Ser-Leu-Ile-Arg-Trp-Lys-Ser-Ser (FSLIRWKSS), which includes the LIRW motif necessary for sorting MAL to GEMs, and whose last five amino acids resemble dilysine-based signals involved in endoplasmic reticulum (ER) retention. We have addressed the influence of the carboxyl-terminal serines in both MAL distribution and incorporation into GEMs. Substitution of the serines by alanine impeded the access of MAL to GEMs and changed its distribution from a perinuclear distribution to an ER pattern. The RWKSS sequence appended to the carboxyl-terminus of CD4 caused retention of the chimera in the ER. Thus, although this pentapeptide can function producing ER retention in other protein context, the presence of the carboxyl-terminal serines in the intact MAL molecule prevents its use as an ER-retention signal.

It has been suggested that internal glycolipid-enriched membranes (GEMs) resistant to detergent solubilization play a role in vectorial transport of proteins and glycolipids to the apical surface in polarized epithelial MDCK cells (1). Although detailed ultrastructural analyses of internal GEM rafts are lacking, the fact that apical and basolateral sorting occurs in the late Golgi has led to the assumption that internal GEMs are specialized subdomains of the *trans*-Golgi-network (1). To function as a route of transport, GEMs require protein sorting machinery that would minimally consist of a set of proteins that carry out the processes of vesicle formation, cargo recruitment, targeting, and fusion with the apical surface (1).

The *MAL* gene was first identified during a search for genes differentially expressed during human T-cell ontogeny (2). More recently, the MAL protein has been identified in myelin-forming cells (3, 4) and in polarized epithelial cells, including the renal MDCK cell line and thyroid cells (5–7). The *MAL* gene encodes a non-glycosylated integral membrane protein of 17 kDa containing multiple hydrophobic segments (2), that, in contrast with most integral membrane proteins, is highly soluble in organic solvents used to extract cell lipids (7). MAL is predominantly distributed in perinuclear vesicles (8), and displays the distinctive biochemical feature of residence in GEMs (6, 7, 9). These facts, together with the observations that MAL is localized at steady state in the apical zone of polarized epithelial cells (7), and that its ectopic expression in insect Sf21 cells provokes a massive *de novo* induction of vesicle formation (10) led to the proposal of MAL as being a possible component of the vesiculation machinery for the apical transport pathway. More recently, depletion of endogenous MAL in intact MDCK has demonstrated that MAL is necessary for normal apical transport and accurate sorting of the influenza virus hemagglutinin (11). This highlights MAL as a component of the machinery for GEM-mediated apical transport.

The carboxyl terminus of MAL has the sequence Phe-Ser-Leu-Ile-Arg-Trp-Lys-Ser-Ser (FSLIRWKSS). The last five amino acids in this sequence (RWKSS) resemble the dilysine-based signals (K/R(X)KXX, where X represents any amino acid) involved in retrieval of type I transmembrane proteins to the endoplasmic reticulum (ER) (12, 13) and the KKXX motif involved in endocytosis (14). Overlapping with that consensus sorting motif, our previous mutational analysis led to the identification of the LIRW tetrapeptide, which is required for MAL sorting to GEMs (15). In the current study we have focused on the role of the last

Abbreviations used: ER, endoplasmic reticulum; GEM, glycolipid-enriched membrane; mAb, monoclonal antibody; PBS, phosphate-buffered saline.

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two carboxyl-terminal serines in both the intracellular distribution of MAL and its incorporation into GEMs using mutants bearing an intact LIRW sequence.

MATERIALS AND METHODS

Materials. Mouse hybridomas producing monoclonal antibodies (mAbs) 9E10 or OKT4 to the 9E10 c-Myc epitope (EQKLISEED) or to human CD4, respectively, were purchased from the American Type Culture Collection. Rabbit polyclonal antibodies to the c-Myc tag were from Santa Cruz Biotechnologies (Santa Cruz, CA). The anti-caveolin-1 and anti-calnexin mAbs were from Transduction Labs (Nottingham, UK). Peroxidase-conjugated goat anti-mouse IgG antibodies were from Pierce (Rockford, IL). Fluorescein- and Texas Red-conjugated antibodies were from Southern Biotech (Birmingham, AL).

Cell culture conditions and DNA constructs. Human epithelial A498 cells (ATCC HB44) were grown on Petri dishes or glass coverslips, in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD), penicillin (50 units/ml) and streptomycin (50 μg/ml), at 37°C in an atmosphere of 5% CO_2, 95% air. The different MAL constructs used in this study were generated by the polymerase chain reaction using oligonucleotide primers with the appropriate modifications. All MAL mutants were tagged with the 9E10 c-Myc epitope at the NH_2-terminus. For DNA constructs expressing CD4, we used primers specific for the 5′ and 3′ ends of the CD4 coding sequence and CD4 cDNA as template (a kind gift from Dr. P. J. Maddon, Columbia University, New York). The MAL RWKSS carboxyl-terminal sequence was added to the COOH-terminus of CD4 (CD4-RWKSS) by amplification with the same 5′ oligonucleotide primer and a 3′ oligonucleotide primer containing the appropriate modification. After cloning into the pSRα eukaryotic expression vector (16), the sequence of inserted product was verified in all of the constructs to eliminate the possibility of amplification errors. Transfections in the A498 cell line were carried out by using Lipofectin reagent following the protocol provided by the manufacturer (Gibco-BRL, Gaithersburg, MD, USA). Selection of stable transfectants was carried out by treatment with 0.5 mg/ml G-418 sulphate for at least 4 weeks after transfection. Drug-resistant cell clones were trypsinized *in situ* with the aid of cloning rings. The clones were screened by immunofluorescence analysis, and those resulting positive for the expression of the indicated MAL proteins were maintained in drug-free medium. Transient transfection of COS-7 cells were done by electroporation using the ECM600 electroporation instrument (BTX, San Diego, CA).

Detergent extraction procedures. GEMs were isolated by standard procedures (17). Cells grown to confluency in 100-mm dishes were rinsed with phosphate-buffered saline (PBS) and lysed for 20 min in 1 ml of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 at 4°C. The lysate was scraped from the dishes with a rubber policeman, the dishes were then rinsed with 1 ml of the same buffer at 4°C, and the lysate was homogenized by passing the sample through a 22-gauge needle. The lysate was finally brought to 40% sucrose in a final volume of 4 ml and placed at the bottom of an 8-ml 5–30% linear sucrose gradient. Gradients were centrifuged for 18 h at 39,000 rpm at 4°C in a Beckman SW41 rotor. Fractions of 1 ml were harvested from the bottom of the tube. For immunoblot analysis, aliquots from each fraction were subjected to SDS-PAGE under reducing conditions and transferred to Immobilon-P membranes (Millipore, Bedford, MA). After blocking with 5% (w/v) nonfat dry milk, 0.05% (v/v) Tween-20 in PBS, membranes were incubated with the indicated antibodies. After several washings, blots were incubated for 1 h with goat anti-mouse IgG antibodies coupled to horseradish peroxidase diluted at 1:5,000, washed extensively, and developed using an enhanced chemiluminescence Western blotting kit (ECL, Amersham Corp.).

Immunofluorescence microscopy. Epithelial A498 cells grown on coverslips were washed twice with PBS, fixed in 3% paraformaldehyde 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 for 1 h with the primary antibody, rinsed several times, and incubated for 1 h with the appropriate fluorescent secondary antibody. For double-label immunofluorescence analysis the procedure was repeated with the second primary and secondary antibodies. After extensive washing, the coverslips were mounted on slides. The cells were photographed with a Zeiss Axioskop photomicroscope using Kodak T-Max 400 film. Primary antibodies included mouse OKT4 (IgG), and anti-calnexin (IgG) mAbs, and rabbit polyclonal antibodies to the c-Myc epitope. Secondary antibodies included Texas Red-conjugated goat anti-mouse IgG; and fluorescein-conjugated anti-rabbit IgG antibodies absorbed against mouse IgG. 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 AND DISCUSSION

Substitution of the two carboxyl-terminal serines by alanine, but not their deletion, impedes access of MAL to GEMs. To study the behavior of MAL mutants at steady state, the analysis has been carried out using A498 epithelial cells that stably express the MAL proteins tagged at their amino terminus with the 9E10 c-Myc epitope. It has been previously demonstrated that this tag does not interfere with the targeting of MAL to GEMs (7, 12). A498 cells were extracted with 1% Triton X-100 at 4°C, and the resulting lysate was subjected to centrifugation to equilibrium in sucrose density gradients following an established protocol (17). Figure 1 shows that both wild type MAL (MAL-FSLIRWKSS) and a mutant lacking the two carboxyl-terminal serines (MAL-FSLIRWK) were exclusively targeted to GEMs consistently with our previous results (15), whereas a mutant bearing the serines substituted for alanine (MAL-FSLIRWKAA) was exclusively found in the soluble fractions. As internal control of the fractionation procedure, aliquots from the same fractions were analyzed with antibodies to either caveolin-1 or calnexin, as representative of integral membrane proteins present in or absent from GEMs, respectively.

The serine residues within the MAL carboxyl-terminal RWKSS pentapeptide prevent retention of MAL in the ER. Figure 2 shows that wild type MAL is localized at steady state on perinuclear structures (A) that are clearly different from the ER pattern of calnexin (C), an ER protein marker. In contrast, substitution of the two carboxyl-terminal serines by alanine (MAL-FSLIRWKAA) led to accumulation of the mutant in the ER (B, D). The observed distribution of MAL-FSLIRWKAA in the ER is probably due to recognition of the RWKAA pentapeptide as an ER retrieval signal. As the mutant MAL-FSLIRWK is able to attain access to GEMs (Fig. 1) and shows a perinuclear vesicular distribution (nor shown), the serine residues prob-

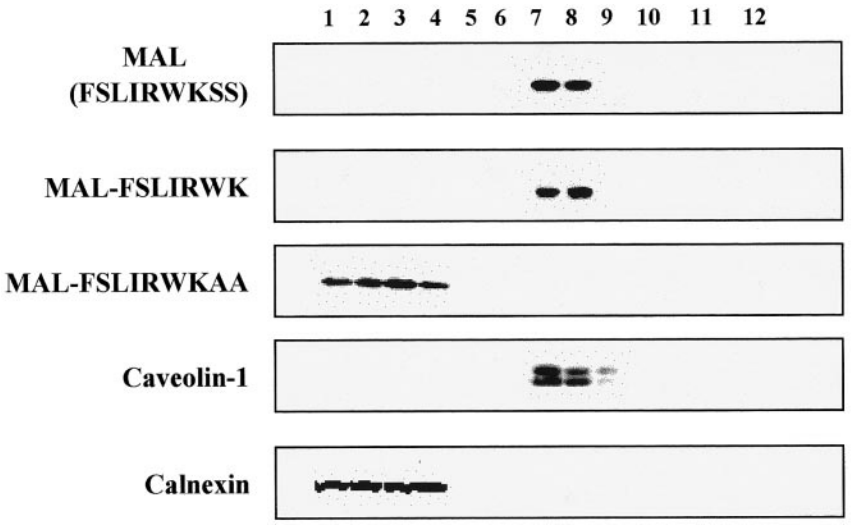

FIG. 1. Substitution of the last two serine residues by alanine, but not their deletion, blocks the incorporation of MAL into GEMs. A498 cells stably expressing MAL tagged with a c-Myc epitope at its NH_2-terminus (MAL-FSLIRWKSS), or variants of this protein in which the carboxyl-terminal serines were deleted (MAL-FSLIRWK) or substituted by alanine (MAL-FSLIRWKAA) were extracted at 4°C with 1% Triton X-100 and centrifuged to equilibrium in sucrose density gradients following standard procedures. 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. The distributions of calnexin, an ER protein that is excluded from GEMs, and of caveolin-1, a GEM-resident protein, were used as control of the fractionation procedure.

ably prevent the recognition of the RWKSS sequence as an ER retrieval signal rather than providing positive signals for ER egress. These results are similar to those of previous reports showing that the two aromatic residues reduce the ER-retrieval efficiency mediated by the dilysine KKFF motif (18).

The MAL RWKSS pentapeptide fused to the carboxyl terminus of CD4 produces retention of the chimera in the ER. Although the RWKSS sequence does not produce retention of MAL in the ER in spite of the resemblance of this pentapeptide to the consensus ER retrieval signal, we investigated whether the fusion of

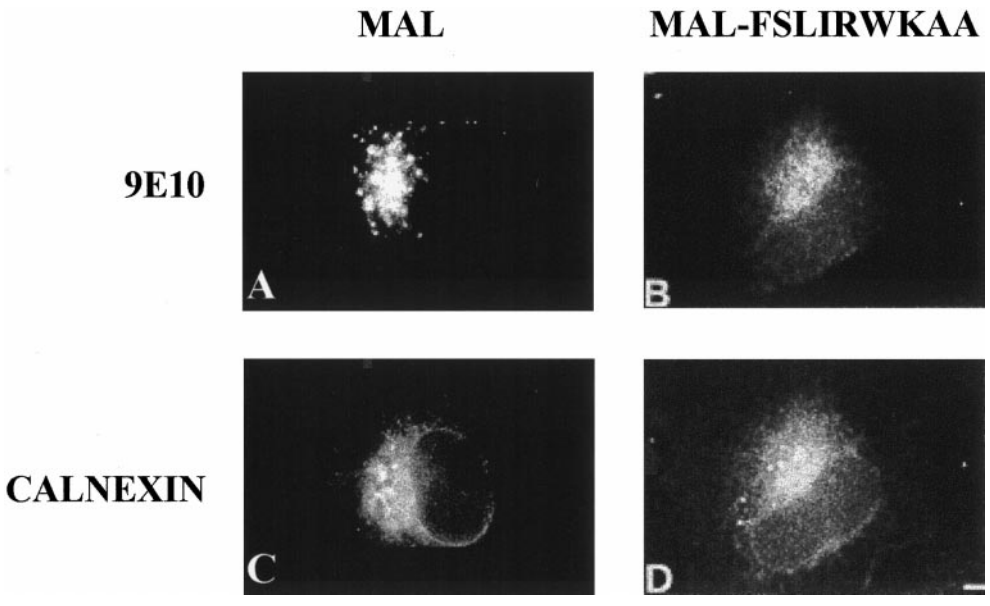

FIG. 2. Replacement of the carboxyl-terminal serines by alanine causes MAL retention in the ER. A498 cells stably expressing MAL-FSLIRWKSS (A, C) or MAL-FSLIRWKAA (B, D) were subjected to double-label immunofluorescence analysis with rabbit polyclonal anti c-Myc antibodies to detect MAL (A, B) and with mouse anti-calnexin mAb to label the ER (C, D). Bar, 3 μm.

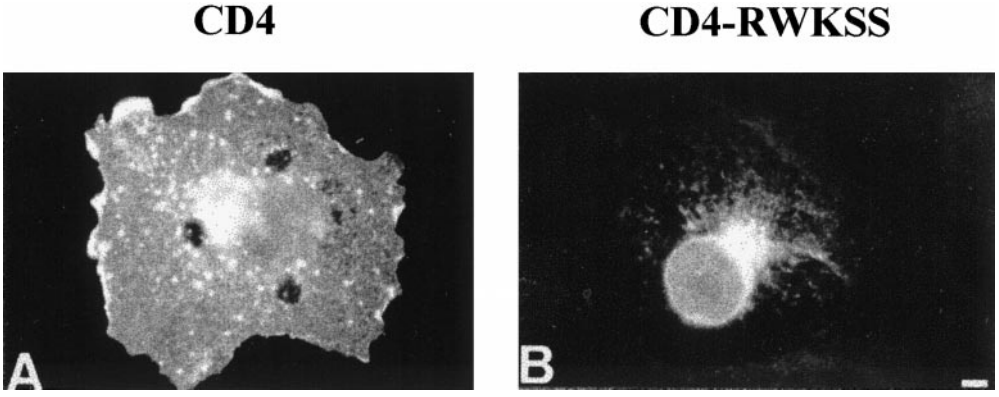

FIG. 3. Appendage of the MAL RWKSS sequence to the carboxyl-terminus of CD4 causes retention of the chimera in the ER. COS-7 cells were transiently transfected with DNA constructs expressing either wild type CD4 (A) or CD4 with the RWKSS sequence appended to its carboxyl terminus (CD4-RWKSS) (B) and subjected to immunofluorescence analysis with anti-CD4 mAb. Bar, 3 μm.

the pentapeptide to the carboxyl terminus of CD4 molecule, chosen as a representative of transmembrane proteins, was able to cause retention of the chimera in the ER. COS-7 cells were transfected with either wild type CD4 or a modified CD4 molecule bearing the RWKSS sequence at the carboxyl terminus (CD4-RWKSS), and subjected to immunofluorescence analysis with anti-CD4 antibodies 24 h after transfection. Figure 3 shows that whereas the wild type CD4 molecule (A) was expressed on the cell surface, the CD4-RWKSS chimera (B) distributed in the ER. Taken together, the results in Figs. 2 and 3 indicate that the presence of the serines in the intact MAL molecule, but not in CD4, prevents the RWKSS being recognized as an ER targeting signal.

Previous reports demonstrated a role for MAL as a component of the integral membrane protein machinery acting in GEMs. Here we have used MAL mutants to assess whether amino acids that are not necessary for the targeting of MAL to GEMs affect the incorporation of MAL into GEMs. The carboxyl-terminal serines, although not required for access to GEMs, play a role in preventing MAL targeting to the ER, probably by interfering with the recognition of the RWKSS pentapeptide as an ER-retrieval [R/K(X)KXX] signal. COP I-coated vesicles have been implicated in retrograde transport of membrane proteins from the Golgi to the ER (19). Coatomer, a major protein complex of the COP I coat, interacts with dilysine-based ER retention signals (20, 21). In addition to its presence in the early secretory pathway, some COP I components have been identified in endosomes (22). This led to the suggestion that COPs might contribute to coat formation for endosomal membrane traffic, although it is not known whether or not dilysine-based motifs are involved in this process (22, 23). In addition to their role as ER retrieval signals, dilysine-based motifs have been also demonstrated to be functional in protein endocytosis (18). Thus, it is possible that COPs and/or dilysine-based signals participate in sorting events in processes other than that of retrieval of transmembrane proteins to the ER. The two phenylalanine residues within the KKFF motif are important for preventing the functioning of this sequence as an ER-retrieval signal and for allowing its use as an endocytosis signal (18). Similarly, the two serines within the RWKSS pentapeptide prevent the use of this sequence for retention of MAL in the ER. Thus, it is plausible that the carboxyl-terminal serines may allow the participation of the RWKSS sequence in post-Golgi MAL trafficking.

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