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The trans-membrane protein p25 forms highly specialized domains that regulate membrane composition and dynamics

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Summary

Trans-membrane proteins of the p24 family are abundant, oligomeric proteins predominantly found in cis-Golgi membranes. They are not easily studied in vivo and their functions are controversial. We found that p25 can be targeted to the plasma membrane after inactivation of its canonical KKXX motif (KK to SS, p25SS), and that p25SS causes the co-transport of other p24 proteins beyond the Golgi complex, indicating that wild-type p25 plays a crucial role in retaining p24 proteins in cis-Golgi membranes. We then made use of these observations to study the intrinsic properties of these proteins, when present in a different membrane context. At the cell surface, the p25SS mutant segregates away from both the transferrin receptor and markers of lipid rafts, which are enriched in cholesterol and glycosphingolipids. This suggests that p25SS localizes to, or contributes to form, specialized membrane domains, presumably corresponding to oligomers of p25SS and other p24 proteins. Once at the cell surface, p25SS is endocytosed, together with other p24 proteins, and eventually accumulates in late endosomes, where it remains confined to well-defined membrane regions visible by electron microscopy. We find that this p25SS accumulation causes a concomitant accumulation of cholesterol in late endosomes, and an inhibition of their motility – two processes that are functionally linked. Yet, the p25SS-rich regions themselves seem to exclude not only Lamp1 but also accumulated cholesterol. One may envision that p25SS accumulation, by excluding cholesterol from oligomers, eventually overloads neighboring late endosomal membranes with cholesterol beyond their capacity (see Discussion). In any case, our data show that p25 and presumably other p24 proteins are endowed with the intrinsic capacity to form highly specialized domains that control membrane composition and dynamics. We propose that p25 and other p24 proteins control the fidelity of membrane transport by maintaining cholesterol-poor membranes in the Golgi complex.

Key words: Golgi, Endosomes, Cholesterol, Raft, Structure, p24 proteins

Introduction

Proteins of the p24 family form a rather unique family of abundant, small (20-24 kDa) type I trans-membrane proteins in the early biosynthetic pathway. They can be sub-divided by sequence homology into 4 sub-families (p23 or delta, p24 or beta, p25 or alpha, and p26 or gamma) (Dominguez et al., 1998; Emery et al., 1999). Mammalian cells contain at least one member of each p23, p24 and p25 subfamily, and three members of the p26 sub-family (Emery et al., 1999). All seem to cycle in the early secretory pathway (Blum et al., 1999; Fullekrug et al., 1999; Rojo et al., 2000), and to localize primarily to the cis-Golgi network (CGN) or the cis side of the Golgi complex (Emery et al., 2000; Fullekrug et al., 1999; Rojo et al., 1997; Stamnes et al., 1995), except p25 (GP25L) which is also abundant in the endoplasmic reticulum (ER) (Dominguez et al., 1998; Wada et al., 1991). They share a predicted exoplasmic coiled-coil domain and a small (12-18 amino acids) cytoplasmically oriented C terminus that carries more or less degenerate sorting motifs required for interactions with COP-I or COP-II (Dominguez et al., 1998; Fiedler et al., 1996; Sohn et al., 1996). Endogenous and ectopically expressed p23 forms stable oligomers that resist solubilization in various detergents (Rojo et al., 2000). Indeed, p24 proteins form hetero-oligomers (Dominguez et al., 1999; Marzioch et al., 1999), and oligomerization via their predicted coiled-coil domains is required for proper localization (Ciufo and Boyd, 2000; Emery et al., 2000).

Several functions, which may not be mutually exclusive, have been attributed to p24 proteins. In yeast, they were proposed to act as cargo receptors for GPI-anchored protein transport from the ER to the Golgi complex (Muniz et al., 2001; Muniz et al., 2000; Schimmoller et al., 1995), but also to play a role in quality control, since Kar2p retention in the ER depends on p24 proteins (Elrod-Erickson and Kaiser, 1996), and since knock-out of some family members triggers the unfolded protein response (Belden and Barlowe, 2001).

Similarly, retention of unfolded proteins in *Caenorhabditis elegans* depends on p24 proteins (Wen and Greenwald, 1999). In mammalian cells, p24 proteins, in particular p23, were proposed to function as COP-I receptors (Sohn et al., 1996), presumably during retrograde transport back to the ER. Interactions with COP-I appear to be regulated by ARF1 (Gommel et al., 2001; Nickel et al., 2002) via p24 (Goldberg, 2000). Finally, we had previously proposed that p23, and presumably other family members, plays a structural, morphogenic role in the organization and/or biogenesis of the Golgi complex (Rojo et al., 1997), and p25 (alpha2p24) was proposed to be involved in the formation of vesicular tubular clusters (VTCs) and ER exit sites (Lavoie et al., 1999). Surprisingly, however, the octuple knockout of all *Saccharomyces cerevisiae* p24 proteins did not cause a general perturbation of the biosynthetic pathway, beyond delayed GPI-anchored protein transport and defective Kar2p retention, leading to the notion that these proteins cannot be essential components of the yeast transport machinery (Springer et al., 2000). In marked contrast, inactivation of the gene encoding p23 in mice is lethal at a very early developmental stage (Denzel et al., 2000).

It is not clear why p23 is essential in mice, while the entire family seems to be dispensable in *S. cerevisiae*. Interestingly, however, the Golgi of *S. cerevisiae* is dispersed, consisting primarily of individual cisternae scattered in the cytoplasm, in contrast to higher eukaryotic cells (Preuss et al., 1992; Rossanese et al., 1999). Thus, the possible role of p24 proteins in organizing the Golgi of high eukaryotic cells may not be essential for *S. cerevisiae* survival. Similarly, the Golgi appears disorganized and dispersed in *Encephalitozoon cuniculi* (Vivares and Metenier, 2001). Sequencing of the complete genome of *E. cuniculi* shows that this simple organism contains genes encoding potential homologues of key proteins involved in transport through the Golgi apparatus, with the notable exception of p24 homologues (Katinka et al., 2001) (Sean Munro, personal communication). The possible existence of links between Golgi organization and p24 proteins has also been suggested in mammalian cells. While p23 is extremely abundant in regions of the cis-Golgi network (CGN) [approx. 12.500 p23 copies/μm^2 (Rojo et al., 1997)], overexpressed p23 protein fails to reach the CGN and accumulates in the ER where it forms regularly shaped tubulo-cisternal membrane clusters (Rojo et al., 2000), like other p24 proteins (Emery et al., 2000). These p23-induced clusters trap endogenous p23, at the expense of the CGN, eventually leading to a fragmentation of the Golgi complex, without measurable inhibition of biosynthetic transport. Similarly, p23 heterozygous mice appear normal, but the morphology of the Golgi complex is altered (Denzel et al., 2000).

The functions of p24 proteins are not easily studied in vivo, since they form oligomeric complexes in membranes (Emery et al., 2000; Fullekrug et al., 1999; Marzioch et al., 1999) and accumulate in the ER when overexpressed alone (Emery et al., 2000; Rojo et al., 2000). In addition, most mutants engineered by us and others either fail to leave the ER, do not behave like wild-type (WT) or they lose their protein interaction capacity. We thus decided to determine whether a member of the p24 family could be targeted to another organelle, so that its intrinsic properties could be studied unambiguously in membranes normally devoid of p24 proteins. Here, we report that inactivation of the canonical KKXX motif in p25 allows transport of a p24 family mutant beyond the Golgi, without affecting its capacity to interact with itself and other family members, demonstrating that p25 functions as an anchor for p24 proteins in the recycling leg of the biosynthetic pathway. We find that p25 is endowed with the intrinsic capacity to form specialized membrane domains at the cell surface in late endosomes, and that formation of such domains in late endosomes disrupts membrane dynamics and cholesterol transport.

Materials and Methods

Cells, reagents and antibodies

Monolayers of HeLa cells were cultured as described previously (Rojo et al., 1997). HeLa cells stably expressing a N-acetylglucosamine-transferase-I (NAGT-I)-GFP chimera were provided by D. Shima and G. Warren (Ludwig Institute for Cancer Research, New Haven) and maintained as described previously (Shima et al., 1997). Rabbit antibodies against p23 (Rojo et al., 1997) and p26 (Emery et al., 2000) were as described; anti-p26 antibodies were a gift from R. Pepperkok (Heidelberg, Germany). We obtained mouse monoclonal antibodies against ERGIC53 (Schweizer et al., 1988) and giantin (Linstedt and Hauri, 1993) from H. P. Hauri (Basel, Switzerland), against β-COP (maD) from T. E. Kreis (Pepperkok et al., 1993) and sheep polyclonal antibodies against GRASP55, p115 and GM130 from F. Barr (Martinsried, Germany). The ASSP aerolysin mutant (Fivaz et al., 2002), chicken polyclonal anti-aerolysin antibody and Cy5-labeled transferrin were provided by G. van der Goot (Geneva, Switzerland). The Cy3-labeled monoclonal antibody (9E10) against the myc epitope was from Sigma Chemical Co. (St-Louis, MO), and the rabbit polyclonal anti-myc antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal antibody against the HA epitope was from BabCO (Richmond, CA) and against the human transferrin receptor from Zymed Laboratories Inc. (San Francisco, CA). Secondary antibodies were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). ps65-CD4 was from Mark Marsh (London, UK) (Sauter et al., 1996).

cDNA and mutagenesis

Construct containing WT and tagged p24 proteins, p23SS and coiled-coil deletion mutants were as described previously (Emery et al., 2000; Rojo et al., 2000). The p24SS and p25SS mutant were generated using pCB6-HA-p24 and pCB6-myc-p25 (Emery et al., 2000) as templates, by classical PCR using the SPP oligonucleotide described by Emery et al. (Emery et al., 2000); 5′-CGG GAT CCT TAA ACA ACG CTG CTG ACT TCA AAA AAT CTC TTC AGG-3′ was used for p24SS and 5′-CGG GAT CCC TAC ACA AGG CTG CTG GCT TCA AAG AAG CTC TTG AGG-3′ for p25SS (*Bam*HI site underlined). PCR products were cloned into *Eco*RI and *Bam*HI sites of pCB6 to form respectively pCB6-HA-p24SS and pCB6-myc-p25SS. All PCR products were verified by sequencing.

A chimera of p23 with the transmembrane region of CD4 (p23CD4TM) was generated by two fusion PCR steps (Ho et al., 1989) using pCB6-myc-p23 and ps65-CD4. First, the transmembrane domain and cytoplasmic tail of p23 was exchanged with those of CD4. Two fragments were produced by PCR on pCB6-myc-p23 with SPP and **TGG ACC ATG TGG GCA GAA CTC TGG TGT TTG TGG A**CT CAT TGG and on ps65-CD4 with 5′-**TCC ACA AAC ACC AGA GTT CTG CCC ACA TGG TCC A**CC CCG-3′ and 5′-CGG GAT CCT CAA ATG GGG CTA CAT GTC TTC-3′ (*Bam*HI site underlined and overlapping sequences in bold). Both fragments were fused by PCR with SPP and the last oligonucleotide described and cloned into *Eco*RI and *Bam*HI sites

of pCB6. This construct was then used in another fusion PCR to restore the cytoplasmic tail of p23, using 5′-**CAG GTA GAA CAC TAG CCC AAT GAA AA**G CAG GAG GCC GG-3′ and SPP to get the first fragment (overlapping sequence in bold). The second fragment was obtained by PCR on pCB6-p23 with 5′-**TTT TCA TTG GGC TAG TGT TCT ACC TG**C GTC GC-3′ and SPM (overlapping sequence in bold). Both fragments were fused by PCR using SPP and SPM. This latter construct was cloned into *Eco*RI and *Bam*HI site of pCB6 to form pCB6-myc-p23-CD4TM. The chimera produced contains the lumenal domain of p23 (from amino acid 1 to 187 of BHKp23), fused to the transmembrane domain of CD4 (amino acids 388-406 of human CD4), fused to the cytoplasmic tail of p23 (aa205-219).

In vivo experiments and indirect immunofluorescence

Cells were transfected 40 hours prior to fixation using the calcium phosphate method (Chen and Okayama, 1987) and processed for immunofluorescence (Rojo et al., 2000; Rojo et al., 1997). To label early and late endosomes, cells were incubated with 3 mg/ml rhodamine-dextran (10 kDa; Molecular Probes) for 5 or 60 minutes, respectively (Gu et al., 1997). To label the plasma membrane, transfected cells were incubated with the indicated antibody for 30 minutes at 4°C in culture medium buffered with 10 mM Hepes pH 7.4, washed with PBS containing 5 mg/ml BSA and processed for microscopy. In some experiments, cells were incubated with the antibody in culture medium for the indicated time before fixation. Patching of raft domains at the plasma membrane with the ASSP mutant of aerolysin (Fivaz et al., 2002) was as described previously (Abrami and van Der Goot, 1999), except that Cy3-labeled monoclonal antibody against myc and Cy5-labeled transferrin were added together with primary chicken antibody against aerolysin, to limit accessibility problems. Cells were visualized using a Zeiss Axiophot fluorescence microscope equipped with a cooled CCD camera (Princeton Instruments), controlled by a Power Macintosh. The IPLab Spectrum 3.1 software (Signal Analytics Corp.) was used for data acquisition.

Time-lapse video microscopy

Time-lapse video microscopy was as described previously (Lebrand et al., 2002), using a Zeiss Axiovert S1000TV fluorescence microscope, a 50 W Hg lamp attenuated by transmission neutral-density filters (Omega Optical, Brattleboro,VT), a CCD camera C4742-95-12NRB (Hamamatsu-City, Japan) and OpenLab Software (Improvision, Coventry, England); exposure time: 100-200 mseconds. Temperature (37°C) and atmosphere (5% CO_2) was controlled with a CTI-3700/37-2-Digital system (PeCon, Erbach-Bach, Germany). Quantification was always done on three separate experiments, and in each experiment motility was analyzed on all vesicles in two separate cells (roughly 100 structures/cell, i.e. 600 per condition).

Electron microscopy

Polyclonal anti-myc antibodies and 10 nm protein A-gold particles (British Biocell International, Cardiff, UK) were mixed and incubated in culture medium for 1 hour at 4°C using a rotary shaker. Then, HeLa cells transfected with p25SS were incubated with the mixture for 3 hours, washed extensively with PBS containing 5% BSA, fixed in 2% glutaraldehyde for 1 hour and processed for microscopy (Stang et al., 1997).

Other methods

Previously published procedures were used for western blot analysis (Rojo et al., 1997) and cholesterol staining with filipin (Kobayashi et al., 1998).

Results

Putative retention motif of p25, but not of p23 or p24 is active in living cells

In previous studies, we showed that p23 and other members of the p24 family are retained in the ER after overexpression (Emery et al., 2000; Rojo et al., 2000), in agreement with others (Blum et al., 1999). Proper targeting of p23 to the cis-Golgi requires p24 co-expression, and vice-versa, and depends on the presence of the conserved coiled-coil domain in each protein, indicating that p23 and p24 must interact with each other, in agreement with biochemical evidence (Ciufo and Boyd, 2000; Emery et al., 2000). Other family members do not substitute for either p23 or p24 but facilitate transport of the p23/p24 pair, arguing for the existence of multiple interactions amongst family members. We found that the degenerate ER retrieval signal of p23 (–KKLIE, Fig. 1) is dispensable for targeting, since the p23 KK to SS mutant (p23SS) behaves like the WT form, and is not transported beyond the Golgi, when expressed alone or together with p24, p25 and p26 [(Emery et al., 2000) and see Fig. 2]. In these experiments, the exoplasmic-oriented N terminus of p23SS was tagged with the myc epitope to unambiguously determine whether the mutant was present at the cell surface using anti-myc antibodies in the absence of cell permeabilization. Similarly, an N-terminal HA-tagged version of p24 harboring –SSVV instead of –RRVV at the C terminus (HA-p24SS) was still retained in the ER (Fig. 2) and both HA-p24SS and myc-p23SS, when co-expressed,

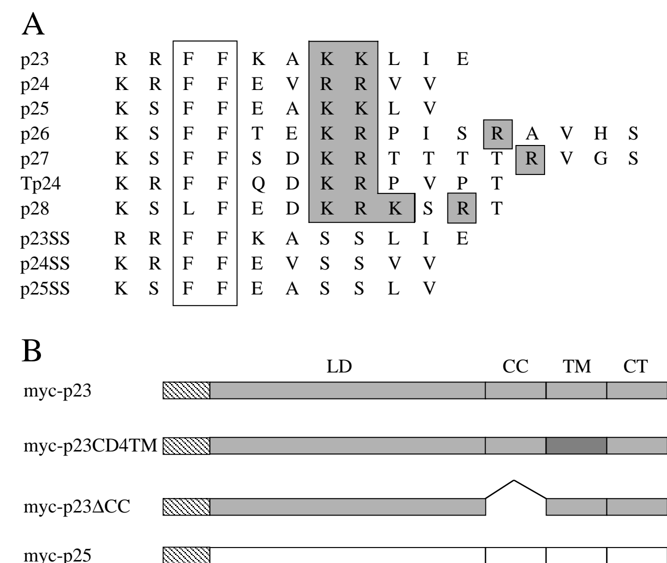

Fig. 1. Outline of p24 proteins and constructs. (A) Alignment of the cytoplasmic tails of p24 proteins and mutants (except chimeras) used in this study. White box: double-phenylalanine motif potentially involved in COP-II binding; gray box: positively charged amino acids, which are proposed to be involved in COP-I binding and retention in the early biosynthetic pathway. We termed p28 a novel putative member of the p24 family identified as a human gene evolutionarily conserved in *Caenorhabditis elegans* by comparative proteomics (accession number: Q9Y3A6). Tp24 was originally named putative T1/ST2 receptor binding protein (see Emery et al., 2000). (B) Schematic representation of p23 and p25 chimera used in this study. LD, lumenal domain; TM, transmembrane domain; CT, cytoplasmic tail. Domains of p23 are in light gray, CD4 in dark gray, and p25 in white.

remained in the early secretory pathway and did not reach the plasma membrane (not shown).

In contrast to the other members of the p24 family, p25 contains the canonical ER retrieval signal KKXX in its cytoplasmic domain (Fig. 1), and is detected in the ER at steady state (Dominguez et al., 1998; Wada et al., 1991). After inactivation of the KKXX motif (KK to SS), an N-terminal myc-tagged version of p25 showed a distribution characteristic of the ER and Golgi complex (Fig. 2), but was also detected at the plasma membrane, particularly in the absence of cell permeabilization (Fig. 2), in agreement with Dominguez et al. (Dominguez et al., 1998). Transport to the plasma membrane was not caused by some imbalance in the stoechiometry of p24 protein complexes after overexpression, since coexpression with any combination of members of the other subfamilies (Fig. 2C and not shown) did not prevent myc-p25SS from reaching the cell surface. The analysis of myc-p25SS steady state distribution showed that, in addition to the ER, Golgi and cell surface labeling patterns, large vesicles were also labeled (Fig. 2), perhaps suggesting that the mutant was endocytosed. When cells were incubated for 16 hours at 37°C with anti-myc antibodies, so that myc-p25SS molecules could bind the antibody when passing by the cell surface, a vesicular pattern characteristic for endosomal compartments was clearly revealed (Fig. 2B). No staining was detected in cells that did not express myc-p25SS, or in cells transfected with proteins that did not reach the cell surface (such as myc-p23SS or HA-p24SS, Fig. 2B), presumably because free antibody molecules taken up by fluid phase endocytosis were eventually degraded in lysosomes. These observations thus show that the KKLV signal of p25 is necessary for p25 retention in the early secretory pathway, in agreement with previous work (Dominguez et al., 1998), and that p25 with an inactive retention motif is transported to the plasma membrane and endocytosed.

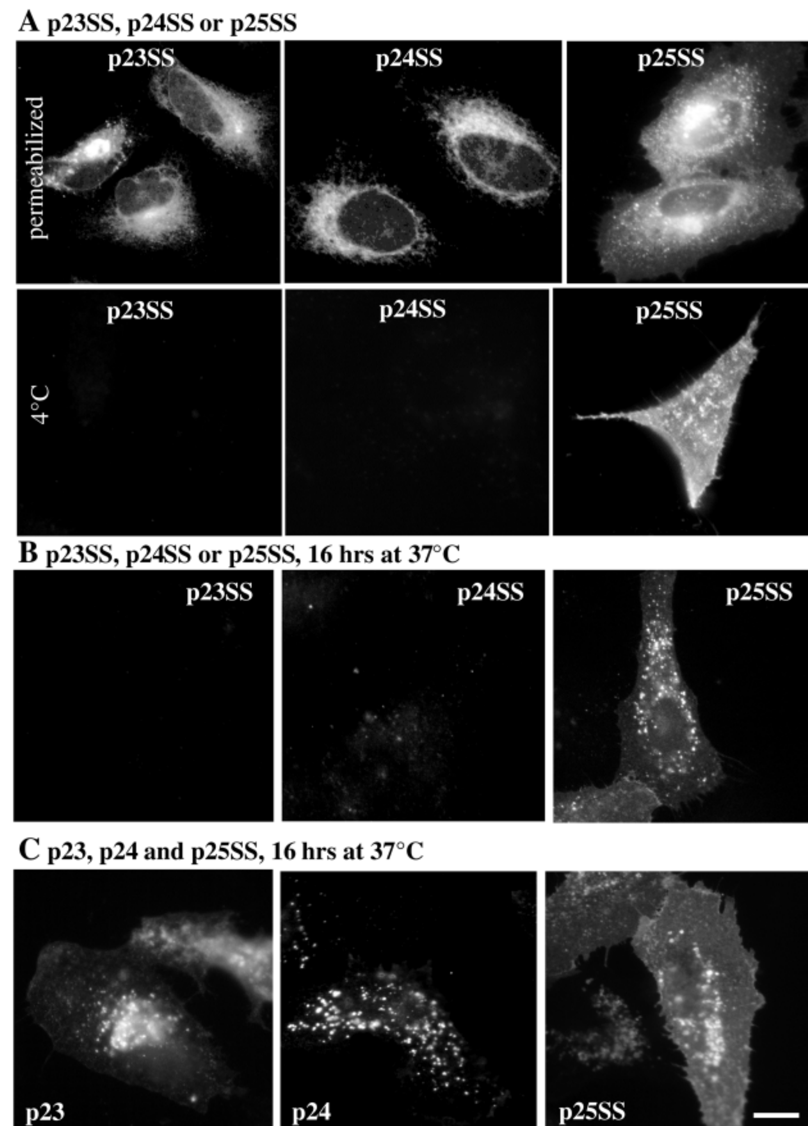

Fig. 2. p25SS transport to post-Golgi membranes. (A) HeLa cells were transfected with myc-p23SS, HA-p24SS or myc-p25SS, as indicated. Then, cells were either permeabilized and the distribution of the indicated proteins was revealed by immunofluorescence using antibodies against myc or HA, or not permeabilized (4°C) and the corresponding antibodies were added to the living cells at 4°C prior to fixation. (B) Cells transfected as in A were incubated for 16 hours at 37°C with antibodies against myc or HA to detect the indicated proteins, and then the distribution of the endocytosed antibodies was revealed by immunofluorescence. (C) Cells were triply transfected with untagged p23, HA-p24 and myc-p25SS. Then, cells were incubated for 16 hours at 37°C with antibodies as in B except that p23 was labeled with anti-lumenal domain antibodies. The distribution of the endocytosed antibodies was analyzed as in B. Scale bar: 10 μm.

The KKXX motif of p25 is responsible for the retention of p24 proteins in the early biosynthetic pathway

We reasoned that p25 may play a role in the retention of other p24 proteins in the early secretory pathway. Indeed, p24 family members interact with each other (Emery et al., 2000; Fullekrug et al., 1999; Marzioch et al., 1999). Moreover, both GMP25 (p25) and p26 were observed at the cell surface after co-transfection with five cDNAs encoding for p24, gp27, p26 and mutant forms of both p25 and p23 with their dibasic motifs mutated to SS (Dominguez et al., 1998). In contrast, both HA-p24SS and myc-p23SS, when expressed alone (Fig. 2A,B) or co-expressed (not shown), did not reach the plasma membrane to any significant extent. However, both WT p23 and WT p24 reached the plasma membrane and were endocytosed, after triple co-transfection with p25SS (Fig. 2C), while WT p24 proteins were never observed beyond the ER and Golgi complex, even after strong over-expression (Emery et al., 2000). In addition, endogenous p23 and p26 were also co-transported with p25SS to the cell surface and endosomes (not shown), much like co-expressed p23 or p24 (Fig. 2C), but the bulk remained in the early secretory pathway, presumably because p24 proteins form relatively stable oligomers in biosynthetic membranes (Rojo et al., 2000).

The co-transport of 24 proteins with p25SS beyond the Golgi complex was highly selective. Indeed, other proteins of the Golgi/CGN (Fig. 3; ERGIC-53, Giantin, GRASP55, p115 and, not shown, NAGTI-GFP) and ER (Fig. 3; calnexin) were not affected by p25SS overexpression. Also, coexpression of p25SS, p23 and p24 did not affect the distribution of GRASP55, which is involved in Golgi retention of p24 proteins (Barr et al., 2001), perhaps because GRASP55 remains bound to other Golgi proteins or because p24 interactions with p25SS are stronger than with GRASP55. Similarly, COPI was not redistributed in cells expressing p25SS, p23 and p24 (Fig. 3), although proteins of the p24 family were proposed to function as COPI receptors (Sohn et al., 1996), perhaps because such interactions are not productive in other organelles (Goldberg, 2000).

Previously, we had shown that the conserved coiled-coil domain present in the lumenal region of p24 proteins is necessary for ER exit and proper targeting to the CGN, presumably because this domain is required for protein-protein interactions amongst family members (Emery et al., 2000). Consistently, p23 and p24 deletion mutants without coiled-coil domain failed to be transported beyond the Golgi in cells overexpressing p25SS (not shown). The transmembrane domain was also proposed to play a role in exit from the ER (Fiedler and Rothman, 1997). We thus constructed a p23 chimera with the WT p23 exoplasmic and cytoplasmic domains flanking CD4 transmembrane domain (p23-CD4TM, see Fig. 1). As expected (Fiedler and Rothman, 1997), the chimera reached the cell surface and was endocytosed (Fig. 4), like p25SS, suggesting that both trans-membrane and coiled-coil domains, but not the degenerate dilysine motif [intact in p23CD4TM (see also Emery et al., 2000)], are necessary for retention in the early biosynthetic pathway. However, in marked contrast to p25SS, the p23CD4TM chimera failed to co-transport other family members to the plasma membrane and endosomes (not shown), presumably because it could not interact efficiently with other p24 proteins, including p25 itself. Altogether, our experiments thus show that p25 plays a crucial role in the specific retention of p24 complexes in the early biosynthetic pathway, and that retention depends on the p25 KKVV motif.

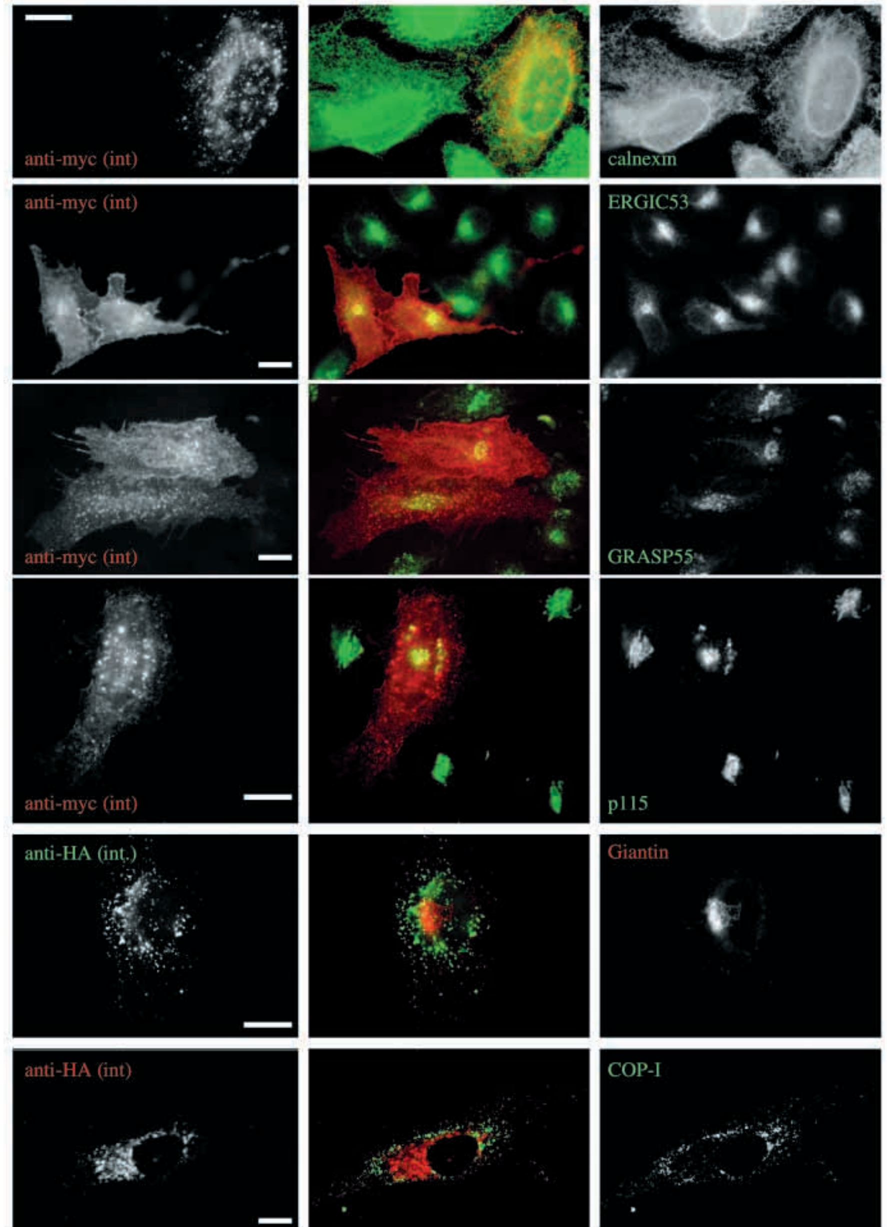

Fig. 3. Localization of early biosynthetic proteins. HeLa cells were transfected with myc-p25SS alone and then incubated with anti-myc antibodies as in Fig. 2B, or triply co-transfected with p25SS, p23 and HA-p24 and then incubated with anti-HA antibodies. The distribution of antibody-tagged myc-p25SS or HA-p24 was then compared with those of the indicated proteins by indirect immunofluorescence. Cells transfected only with myc-p25SS are shown in the panels that also illustrate the distribution of calnexin, ERGIC53, GRASP55 and p115, while triply co-transfected cells are shown in the panels that also illustrate the distribution of giantin and COP-I. The central column shows merged micrographs, color-coded as indicated. Scale bars: 10 µm.

p25 with an inactive ER retention motif is transported to late endosomes

Next, we analyzed the sub-cellular distribution of endocytosed myc-p25SS using FITC-dextran, as a fluid phase tracer. After a 5 minute incubation at 37°C, when the tracer had reached early endosomes, colocalization with p25SS was minimal (not shown). Similarly, little colocalization was observed between p25SS and the human transferrin receptor (TfR) (not shown). In contrast, the majority of p25SS-labeled structures containing dextran internalized for 60 minutes (Fig. 4, upper panels), indicating that the bulk of p25SS had reached late endocytic

compartments. In addition, p25SS was found in the close vicinity of both Lamp1, a major glycoprotein of late endosomes and lysosomes (Lewis et al., 1985), and lyso-bisphosphatidic acid (LBPA), an unusual phospholipid exclusively present in late endosomes and involved in both protein and lipid transport through this compartment (Kobayashi et al., 1998). However, p25SS- and Lamp1-positive structures, although closely juxtaposed, did not seem to overlap, particularly in cells expressing high levels of p25SS (see high magnification views in Fig. 4), with Lamp1 apparently surrounding p25SS. The same type of closely juxtaposed labeling patterns was observed with myc-p25SS and LBPA, although the overlap was significantly higher than with Lamp1 (Fig. 4).

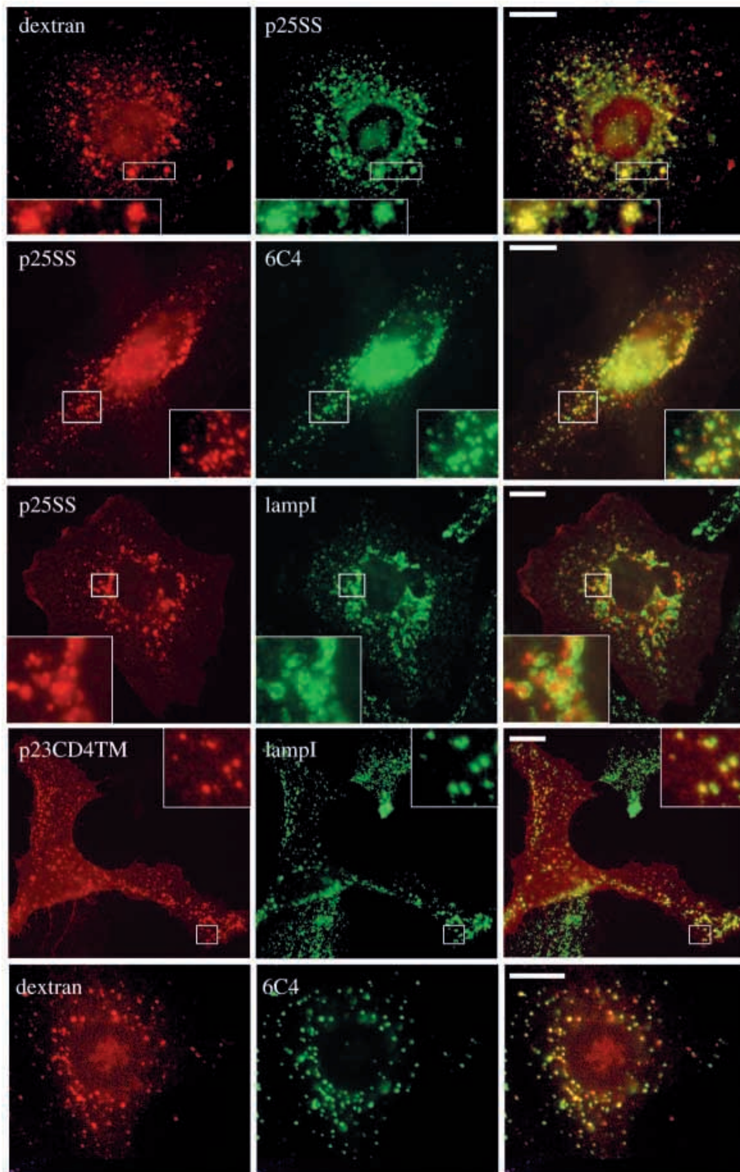

Fig. 4. Distribution of p25SS in the endocytic pathway. Cells were transfected with myc-p25SS and then incubated with rabbit anti-myc antibodies, as in Fig. 2B. Where indicated, rhodamine-labeled dextran was added to the incubation medium during the last 60 minutes of incubation. To label late endosomes, 5 μg/ml monoclonal anti-LBPA antibody (6C4) were co-endocytosed with anti-myc antibodies, to limit accessibility problems within late endosomes (a non-relevant control antibody does not accumulate intracellularly [not shown and (Kobayashi et al., 1998)]. At this low dose, 6C4 does not interfere with trafficking. Then, endocytosed antibodies were revealed as in Fig. 2B using appropriate secondary antibodies. The distribution of antibody-tagged p25SS was compared with that of endocytosed dextran or anti-LBPA antibodies, or to Lamp1 (using anti-Lamp1 antibodies), as indicated. The bottom row shows the distribution of endocytosed dextran and LBPA in untransfected control cells; the right column shows the merged images. Scale bars: 10 μm.

At high expression levels, accumulation of p25SS in late endocytic compartments seemed to affect their organization, since these appeared aggregated, often swollen and enlarged, when compared to control cells (Fig. 4, bottom panels). These effects were not caused by antibody-mediated cross-linking of p25SS molecules, since Lamp1-positive structures were also aggregated and enlarged in the absence of antibody internalization (transfected cells were then identified by labeling p25SS present at the cell surface immediately before fixation; not shown), and since internalization of antibodies against Lamp1 (Kobayashi et al., 1998) or CD63/Lamp3 (Kobayashi et al., 2000) did not cause late endosome swelling, even when used at much higher doses. Moreover, effects were specific to p25SS, since p23-CD4TM also reached late endocytic compartments, but did not affect their morphology even at very high expression levels, and co-localized almost perfectly with Lamp1 (Fig. 4). These data thus show that endocytosed p25SS is transported to late endosomes and suggest that, upon accumulation, p25SS alters late endosome morphology and segregates away from endogenous proteins into separate membranes or membrane domains.

Overexpressed p25SS accumulates within late endocytic membrane domains

To analyze the distribution of myc-p25SS within late endocytic membranes, myc-p25SS was labeled with endocytosed anti-myc antibodies and protein A-gold, and cells were then processed for electron microscopy. Large late endocytic structures with a characteristic multivesicular appearance were heavily labeled, but the gold particles were not distributed evenly on the membrane. The labeling was restricted to relatively small electron-dense regions that often appeared in appropriate section planes continuous with, or contained within, much larger structures (Fig. 5A). Moreover, gold particles were abundant within internal membranes, presumably accounting for the higher degree of p25SS colocalization with LBPA than with Lamp1, since LBPA and Lamp1 are restricted to late endosome internal and limiting membranes, respectively (Kobayashi et al., 1998). The restricted distribution of p25SS within late endosomes is consistent with the propensity of p24 proteins to form oligomers and to accumulate within specialized membrane domains in the early biosynthetic pathway (Rojo et al., 2000; Rojo et al., 1997).

To further investigate the capacity of p25SS to segregate away from endogenous endosomal proteins,

we made use of the ASSP mutant of the pore-forming toxin aerolysin (Fivaz et al., 2002). Normally, the pro-toxin binds cell surface GPI-anchored protein and, after cleavage by furin, the mature toxin forms pores in the plasma membrane. The ASSP mutant carries an engineered disulfide bridge between the activation peptide and the toxin core, thereby preventing toxin activation (Fivaz et al., 2002). In the latter study, it was shown that ASSP is transported to late endosomes, at least in some cell types, where it is reduced and activated, and that toxin activation causes a highly specific and dramatic vacuolation of these endosomes. After ASSP treatment of p25SS-expressing cells, Lamp1 was re-distributed evenly on the membranes of large ASSP-induced vacuoles (Fig. 5B), thus demonstrating that toxin transport to late endosomes was not impaired by p25SS expression. In contrast to Lamp1, p25SS labeling remained confined to small, well-defined spots, and these were often observed at the internal periphery of large Lamp1-positive vacuoles (Fig. 5B, see high magnification views: Lamp1 is in green and p25SS in red). These data show that p25SS, presumably as high order oligomers, causes the formation of robust membrane domains within late endocytic compartments, without inhibiting transport from early to late endosomes, and also demonstrate that these p25SS domains are well segregated from Lamp1, one of the major membrane proteins of external membranes of late endocytic compartments.

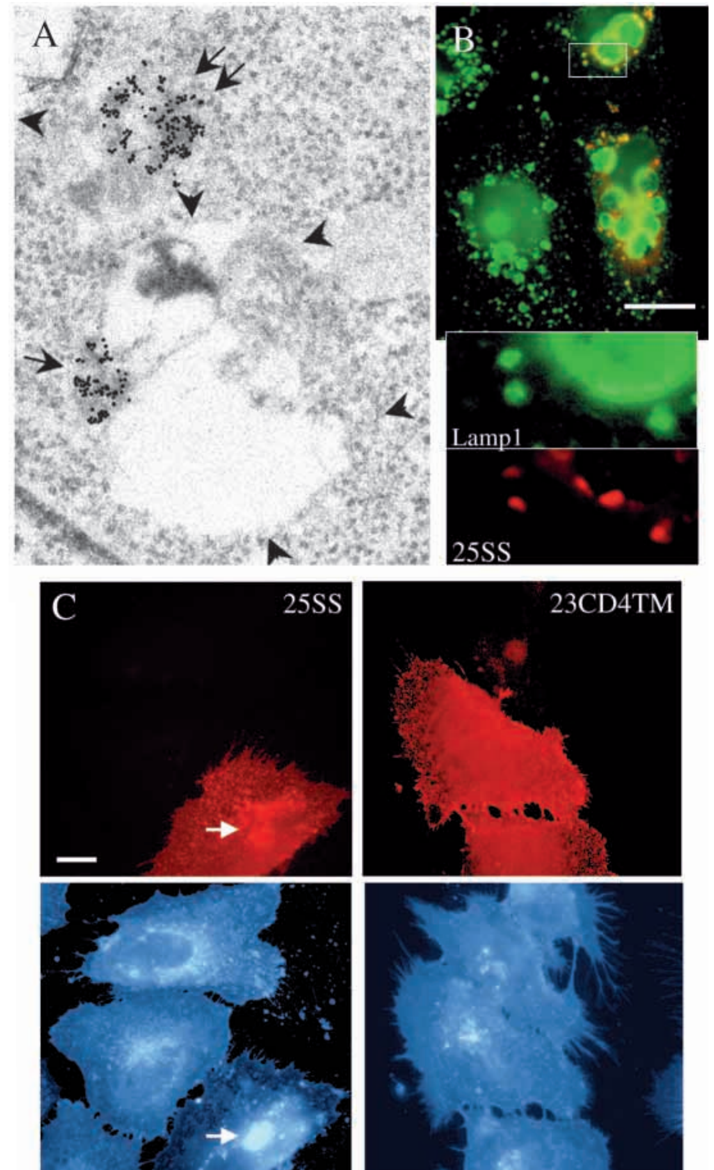

Fig. 5. Clusters in endosomes cause a cholesterol storage defect. **(A)** Cells were transfected with myc-p25SS, incubated with anti-myc antibodies, as in Fig. 2B, and processed for electron microscopy to reveal the distribution of antibody-tagged p25SS clusters (arrows). The membrane of typical late endosomes is outlined by arrowheads. **(B)** HeLa cells were transfected with myc-p25SS and then treated with 0.5 µg/ml ASSP mutant of aerolysin for 16 hours at 37°C. The distribution of antibody-tagged p25SS was then compared to that of Lamp1, as in Fig. 4. The toxin mutant causes late endosome vacuolation. **(C)** Cells were transfected with myc-p25SS or myc-p23-TMCD4 and processed for indirect immunofluorescence using anti-myc antibodies, and filipin to reveal cholesterol distribution. The arrow points to cholesterol accumulation in a p25SS-transfected cell. Scale bars: 10 µm.

p25SS-domains cause cholesterol accumulation and inhibit late endosome dynamics

Evidence is accumulating that late endocytic membranes play a critical role in cholesterol transport, a process impaired in the cholesterol storage disorder Niemann-Pick type C (NPC) (Kobayashi et al., 1999). While in control cells, cholesterol was found primarily at the cell surface and in internal vesicles, presumably corresponding to early/recycling endosomes and the TGN, as expected (Khelef et al., 2000) cells overexpressing p25SS accumulated large amounts of cholesterol in the perinuclear region (Fig. 5C). In these structures, cholesterol colocalized largely with Lamp1 (Fig. 6A), much as in NPC cells (Kobayashi et al., 1999). By contrast, p23-CD4TM overexpression did not affect cholesterol distribution (Fig. 5C). When comparing the distribution of p25SS itself and accumulated cholesterol, however, both were present in closely juxtaposed, and yet distinct, structures (Fig. 6A), like p25SS and Lamp1 (Fig. 4), suggesting that p25SS-rich membrane domains exclude cholesterol.

We and others recently showed that the motility of late endocytic compartments is inhibited in NPC cells and by treatments that mimic NPC (Lebrand et al., 2002; Zhang et al., 2001). In the studies reported here, we had used a GFP-tagged version of Lamp3/CD63 that is properly targeted to late endocytic compartments and colocalizes with both LBPA and Lamp1 to show that CD63-GFP-labeled membranes form highly dynamic tubulo-vesicular networks and that these dynamic properties are inhibited by cholesterol accumulation. Cells were thus cotransfected with CD63-GFP and p25SS, incubated with Cy3-tagged anti-myc antibodies to identify transfected cells, and analyzed by video-microscopy (exposure time was 100 mseconds, and frames were captured every second over a 30-second period). We found that motility was strongly inhibited in cells expressing p25SS. Individual vesicles then exhibited mostly Brownian-type motions (Fig. 6B), in marked contrast to control cells (quantification in Fig. 6C). Effects on motility were specific for late endocytic

compartments, since the brefeldinA-induced redistribution of the Golgi complex into the ER, which depends on microtubules and motors (Lippincott-Schwartz et al., 1990), was not affected by p25SS overexpression (not shown). Moreover, the degree of inhibition was very similar to that observed after cholesterol accumulation in the absence of p25SS overexpression (Lebrand et al., 2002). These data thus show that p25SS accumulation in late endocytic compartments interferes with their functions in cholesterol transport and inhibits their dynamic properties.

p25SS forms structural membrane domains

Since p25SS overexpression caused cholesterol accumulation and yet p25SS-rich domains seemed to exclude cholesterol, we made use of the p25SS molecules present at the plasma membrane to investigate in more detail how the mutant is distributed in membrane domains that are or not enriched in cholesterol. Indeed, cholesterol is enriched within lipid rafts at the cell surface (Ikonen, 2001) and rafts can be conveniently labeled with aerolysin, since this toxin uses receptor GPI-anchored proteins that selectively accumulate in rafts (Schiavo and van der Goot, 2001). We used the ASSP aerolysin mutant, since it binds GPI-anchored proteins like the WT toxin (Fivaz et al., 2002). After ASSP binding to the cell surface, raft clustering was induced by anti-aerolysin antibodies (Abrami and van der Goot, 1999), so that clusters separated away from the glycerophospholipid regions of the bilayer and become visible by light microscopy (Harder et al., 1998). Then, p25SS and ASSP exhibited a mutually exclusive distribution (Fig. 7A). Even more strikingly, the distributions of p25SS and the transferrin receptor (labeled with Cy5-transferrin), a classical marker of the glycerophospholipid regions of the bilayer, were also mutually exclusive, the plasma membrane appearing like a mosaic of rafts, p25SS domains and regions containing the transferrin-receptor. By contrast, p23CD4TM showed a much wider distribution, and even partially distributed to rafts (Fig. 7B). Consistently, p25SS was not randomly distributed at the cell surface, but clustered within defined membrane regions (Fig. 7C). Hence, p25SS did not distribute evenly on the cell surface, nor did it partition into rafts, but it formed well-defined membrane domains that exclude the transferrin receptor.

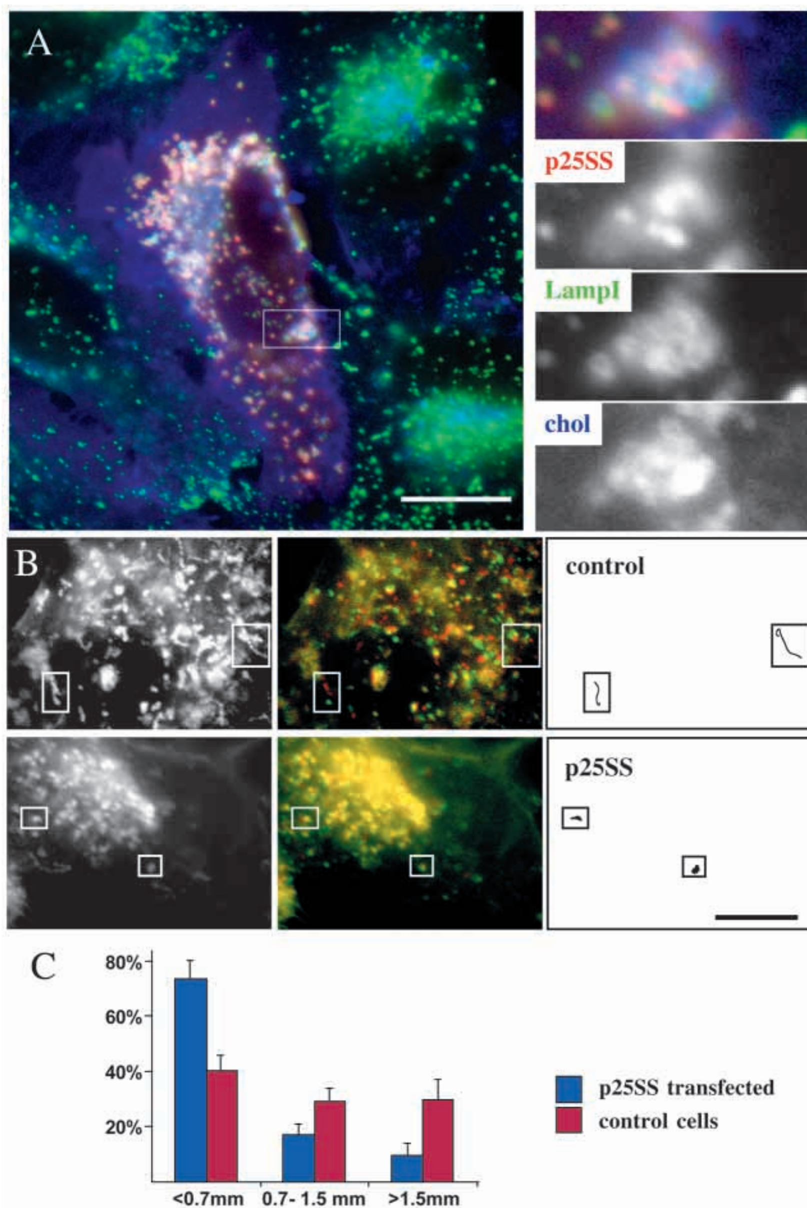

Fig. 6. Cholesterol accumulation and motility inhibition. (A) Cells were transfected with myc-p25SS, incubated with anti-myc antibodies, as in Fig. 2B, and then processed for triple-channel immunofluorescence. The distribution of antibody-tagged p25SS (red) was compared to that of Lamp1 (green) and cholesterol (blue, directly revealed with filipin) in a merged image. A high magnification view of the outlined area is shown in color for each compound, emphasizing the differences in the distribution of p25SS, Lamp1 and cholesterol. (B) Cells were transfected with CD63-GFP alone or together with myc-p25SS. Transfected cells were identified with anti-myc antibodies added to the living cells (not shown). The motility of late endosomes containing CD63-GFP was analyzed (Lebrand et al., 2002) by collecting images (200 mseconds exposure time) every second over a 25-second period. Then, all images were stacked. When represented in this manner, a moving object shifts position, thus creating a series of overlapping or closely associated spots that reveals its track (left panels), as in the control cells. Initial and final positions were color-coded after electronic conversion of the first and last pictures in the sequence to red and green, respectively, so that a moving object appears both red and green, and an immobile object yellow (middle panels). Examples of the traces of individual elements are shown, and highlighted by boxes in the right panels. (C) In cells co-transfected with CD63GFP and p25SS, as in B, the direct distances between initial and final positions of CD63GFP-labeled vesicles were quantified after 25 seconds (and not the actual trajectory followed by vesicles), as indicated (700 vesicles were analyzed and standard deviations are shown). Scale bars: (A,B) 10 μm.

Discussion

p25 anchors p24 proteins in the early secretory pathway

While major progress has been made in the characterization of p24 sorting motifs, mainly by using biochemical approaches in vitro, it has been difficult to study the functions of these proteins in vivo, since they form hetero-oligomers and are retained in the endoplasmic reticulum when expressed ectopically. Also, p23 knockout in mice was lethal at a very early stage (Denzel et al., 2000), while the octuple knockout of all family members in yeast showed only a mild phenotype (Springer et al., 2000). Here, we report that inactivation of the canonical KKXX motif in p25 allows the p25KK to SS mutant to be transported beyond the Golgi to the cell surface and endosomes, in contrast to the corresponding mutations in the degenerate KKXX motif of p23 or p24. Strikingly, p25SS expression also overcomes the mechanisms retaining p24 proteins in the early secretory pathway, without affecting other Golgi proteins.

Since p25 is the only protein of the p24 family containing a canonical di-lysine motif, and since this motif is likely to play a major role in the retention of p24 hetero-oligomers, it seems attractive to conclude that such oligomers are transported beyond the Golgi if they incorporate the p25SS mutant instead of p25. We were unable to show biochemically whether p25SS molecules transported beyond the Golgi formed oligomers, because p25SS is also present in the early secretory pathway (not shown; see Fig. 2), presumably associated to oligomers containing endogenous p25, and because p25SS accumulation in endosomes alters their fractionation properties. However, several lines of evidence strongly suggest that p25SS has retained the capacity to form oligomers in post-Golgi membranes. Not only are p24 proteins co-transported with p25SS to the plasma membrane and endosomes, but this co-transport depends on the conserved coiled-coil domain, which is required for protein-protein interactions (Emery et al., 2000). Moreover, we find that p25SS forms microscopically visible domains at the plasma membrane and in endosomes, which presumably correspond to oligomeric clusters of p24 proteins. We thus conclude that p25 plays a crucial role in the retention of p24 complexes in early biosynthetic membranes, presumably by anchoring p24 oligomers in the COP-I recycling pathway.

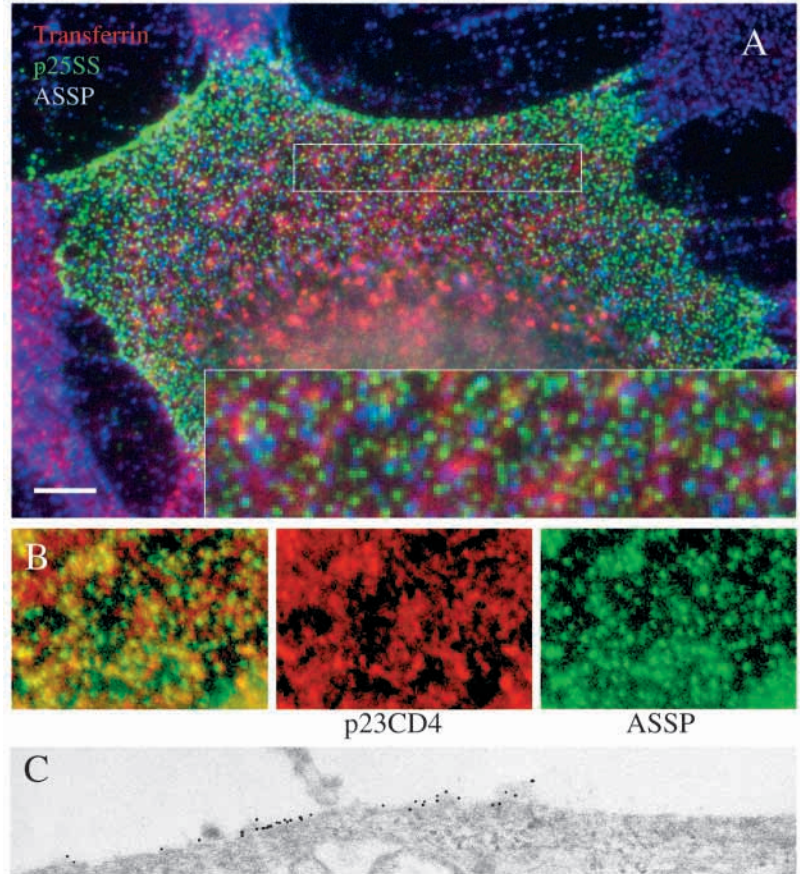

Fig. 7. Cell surface clusters. (A) Cells were transfected with myc-p25SS, incubated without permeabilization at 4°C with the ASSP mutant of aerolysin. Then, cells were treated simultaneously with chicken anti-aerolysin antibodies, Cy3-anti-myc antibodies (color-coded in green) and Cy5-transferrin (color-coded in red), to avoid possible accessibility problems due to raft clustering. Finally, raft domains were further clustered and visualized using anti-chicken secondary antibodies (color-coded in blue). An analysis by triple-channel fluorescence shows the distribution of each marker in a transfected cell, and the inset shows a high magnification view of the outlined area. (B) As in A, except that cells were transfected with p23-CD4TM, and transferrin was omitted. A high magnification view of the plasma membrane shows the distribution of ASSP, p23-CD4TM and the merged image (left). (C) Cells transfected with p25SS were processed for electron microscopy. The micrograph shows a high magnification view of the plasma membrane with immunogold labeling of p25SS. Scale bar: 2 μm.

Membrane domains

At the cell surface, overexpressed p25SS segregates away from rafts and the transferrin receptor and distributes within microscopically visible clusters. Eventually, the mutant protein accumulates in late endosomes within well-defined morphological regions, rather than being efficiently transported to lysosomes and degraded. These p25 domains or clusters are robust, since they resist late endosome vacuolation induced by a mutant form of the pore-forming toxin aerolysin, in contrast to the major late endosomal and lysosomal glycoprotein Lamp1. Endosomal clusters are also reminiscent of those observed when overexpressed p24 proteins, unable to reach their normal destination, are trapped in the endoplasmic reticulum (Rojo et al., 2000), which is consistent with the notion that these proteins tend to self-assemble into higher order structures. Altogether, our observations show that p25SS causes the formation of stable clusters or domains, presumably p24 protein oligomers, in post Golgi membranes, underscoring the notion that the oligomerization process is an intrinsic property of this protein family that does not depend on the membrane composition.

Our analysis also shows that p25SS domains seem to exclude not only other trans-membrane proteins but also cholesterol and to cause a cholesterol storage defect in

endosomes. Clearly, the oligomerization of p25SS, and presumably other p24 proteins in the plane of the membrane, may simply exclude other trans-membrane proteins from p25SS clusters. Indeed, a mutant p23 containing the trans-membrane segment of CD4, which is transported to the cell surface and endocytosed like p25SS, does not efficiently co-transport other p24 proteins or exclude other membrane components, and does not form clusters at the cell surface or in endosomes, in contrast to p25SS. This experiment also suggests that the trans-membrane domain is involved in p24 protein interactions and oligomerization, in addition to the coiled-coil domain (Emery et al., 2000).

Cholesterol and motility

Why do p25SS clusters exclude cholesterol and also cause cholesterol accumulation in late endocytic vesicles? Exclusion may result from limited cholesterol diffusion into the tightly packed trans-membrane alpha-helices of p25SS oligomers. It has also been proposed that short trans-membrane segments, like those found in p25 and other p24 proteins (18-19 residues), are best accommodated by membranes at early steps of the biosynthetic pathway and longer segments within wider, cholesterol-rich, post-Golgi membranes (Munro, 1995). Formation of p25SS oligomers may compress the bilayer and, in this process, exclude cholesterol.

The simplest interpretation for the observed accumulation of cholesterol is that p25SS clusters inhibit cholesterol transport by causing a traffic jam in late endocytic compartments (Gruenberg, 2001). It is possible that p25SS-rich regions interfere with cholesterol transport by sequestering a key component, including perhaps LBPA itself, since this lipid is present within p25SS regions (Fig. 4) and is involved in cholesterol export from late endosomes (Kobayashi et al., 1999). It is also attractive to consider that the exclusion of cholesterol from membrane regions that accumulate oligomeric p25SS causes a concomitant increase in the cholesterol content of neighboring membranes beyond their capacity, eventually resulting in an NPC-like phenotype. Indeed, several lines of evidence indicate that amounts of cholesterol tolerated by late endosomes is limited (Simons and Gruenberg, 2000). In any case, our observations that p25SS causes cholesterol accumulation in late endocytic vesicles and inhibits their motility fits very nicely with our previous findings that the motility of late endocytic vesicles is inhibited by cholesterol accumulation, including in NPC cells (Lebrand et al., 2002). In the latter study, we found that motility is controlled by cholesterol levels via the small GTPase Rab7. Cholesterol accumulation increases amounts of membrane-associated Rab7, which in turn regulates motor activity. Consistently, we find that p25SS overexpression increases levels of membrane-associated Rab7 (not shown), presumably via cholesterol accumulation.

The biosynthetic pathway

Proteins of the 24 family not only form oligomers, which seem to be robust, but are also very abundant in early biosynthetic membranes, with p23 alone accounting for ≈12.500 copies in membrane domains at the cis side of the Golgi complex (Rojo et al., 1997). It thus seems logical to believe that they are involved in membrane organization, perhaps by shaping tubulo-vesicular clusters into flattened cisternae at the cis side of the Golgi, consistently with observations that the Golgi organization is partially disrupted in p23 heterozygous cells and in cells overexpressing p23 (Denzel et al., 2000; Rojo et al., 2000). Interestingly, the matrix proteins (p115, GM130, Giantin and both GRASP55 and GRASP65) were shown to play a structural role in Golgi organization and recent studies reveal that some matrix proteins interact with p24 proteins (Barr et al., 2001). In addition to such a structural role, p24 proteins may be directly involved in protein and lipid sorting. A cholesterol-exclusion mechanism, similar to that observed at the plasma membrane and in endosomes, may well operate in the Golgi complex. The p25-dependent incorporation of p24 protein oligomers in retrograde transport vesicles or tubules may preclude the incorporation of both cholesterol and newly synthesized proteins with long trans-membrane segments destined for post-Golgi membranes. Indeed, raft-like membranes have recently been isolated from the Golgi (Gkantiragas et al., 2001), but COP I vesicles that contains large amount of p24 proteins seems to be devoid of cholesterol (Brugger et al., 2000). Such a mechanism would contribute to an explanation of how a cholesterol gradient is maintained throughout the early biosynthetic pathway and the Golgi complex (Lange et al., 1999). It would also explain how the fidelity of forward transport is ensured by limiting the back-flow of newly synthesized proteins destined for post-Golgi membranes.

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