

# Transmembrane domains control exclusion of membrane proteins from clathrin-coated pits

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## Summary

Efficient sorting of proteins is essential to allow transport between intracellular compartments while maintaining their specific composition. During endocytosis, membrane proteins can be concentrated in endocytic vesicles by specific interactions between their cytoplasmic domains and cytosolic coat proteins. It is, however, unclear whether they can be excluded from transport vesicles and what the determinants for this sorting could be. Here, we show that in the absence of cytosolic sorting signals, transmembrane domains control the access of surface proteins to endosomal compartments. They act in particular by determining the degree of exclusion of membrane proteins from endocytic clathrin-coated vesicles. When cytosolic endocytosis signals are present, it is the combination of cytosolic and transmembrane determinants that ultimately controls the efficiency with which a given transmembrane protein is endocytosed.

**Key words:** Transmembrane domains, Clathrin, Endocytosis, Exclusion, Vesicles, Sorting

## Introduction

Membrane proteins are constantly transported along the secretory and endocytic pathways in eukaryotic cells. To maintain the specific composition of each compartment traversed by this flow of membrane, active sorting is essential at each step of transport. During the formation of a transport vesicle carrying material from a donor to an acceptor compartment, differential sorting can be achieved by two means: concentration or exclusion. Proteins that are concentrated in transport vesicles are depleted from the donor compartment and enriched in the acceptor compartment. Proteins that are excluded from transport vesicles are restricted to the donor compartment. Both mechanisms are not mutually exclusive and might act in concert to ensure efficient sorting of proteins.

Concentration of proteins in transport vesicles has been a subject of very intense scrutiny in the last decades. It is well established that specific sets of cytosolic proteins form coats around budding vesicles and concentrate certain membrane proteins in the vesicle by interacting with their cytosolic domains. This mechanism is, for example, at play during the formation of endocytic vesicles, where adaptor proteins of the clathrin coat concentrate transmembrane proteins bearing cytosolic endocytosis signals (Bonifacino and Traub, 2003; Sorkin, 2004).

Much less is known about exclusion of proteins from transport vesicles. In one of the best studied examples, the formation of endocytic vesicles, efficient exclusion of some markers from coated pits has been reported (Bretscher et al., 1980; Nichols, 2003; Pelchen-Matthews et al., 1992), suggesting that active concentration of specific cargo in coated pits causes passive steric exclusion of non-selected proteins. However, conflicting results were reported concerning the fate of membrane proteins bearing no cytosolic endocytosis signals. Only a modest exclusion was observed for a mutant growth hormone receptor devoid of endocytic signals (Sachse et al., 2001). Even more striking, the concentration of a mutant CD4 with a deleted cytosolic domain was found to be

identical inside and outside clathrin-coated pits (Pelchen-Matthews et al., 1991). Similarly, some GPI-anchored proteins were found at the same concentration inside and outside clathrin-coated pits (Mayor and Maxfield, 1995; Mayor et al., 1998). These results suggest that exclusion from clathrin-coated pits cannot be accounted for solely by steric hindrance and that unidentified determinants control the degree of exclusion of individual membrane proteins from coated pits.

Transmembrane domains (TMDs) of integral membrane proteins have been shown to contain determinants controlling sorting in the secretory pathway (Bonifacino et al., 1991; Machamer et al., 1993; Munro, 1991). A few observations suggest that TMDs might also play a role in the sorting of membrane proteins in the endocytic pathway. In budding yeast, it has been reported that transport to the vacuole or to the cell surface was at least partially determined by TMDs: proteins with long TMDs were targeted to the cell surface; those with shorter TMDs were transported to the vacuole (Rayner and Pelham, 1997; Roberts et al., 1992). In mammalian cells, alterations in the TMD of the transferrin receptor were shown to affect its transport in the endocytic pathway (Zaliauskiene et al., 2000). More recently, it was shown that a viral protein (HCMV UL16) was targeted to endosomal compartments partly by its TMD (Vales-Gomez and Reyburn, 2006). Besides the fact that only a few observations are available about sorting of TMDs in the endocytic pathway, the precise transport step at which TMDs might be sorted remained undefined or was proposed to be intracellular, for example, transport from endosomes to the cell surface (Zaliauskiene et al., 2000) or from the Golgi apparatus to endosomes (Rayner and Pelham, 1997; Roberts et al., 1992).

## Results

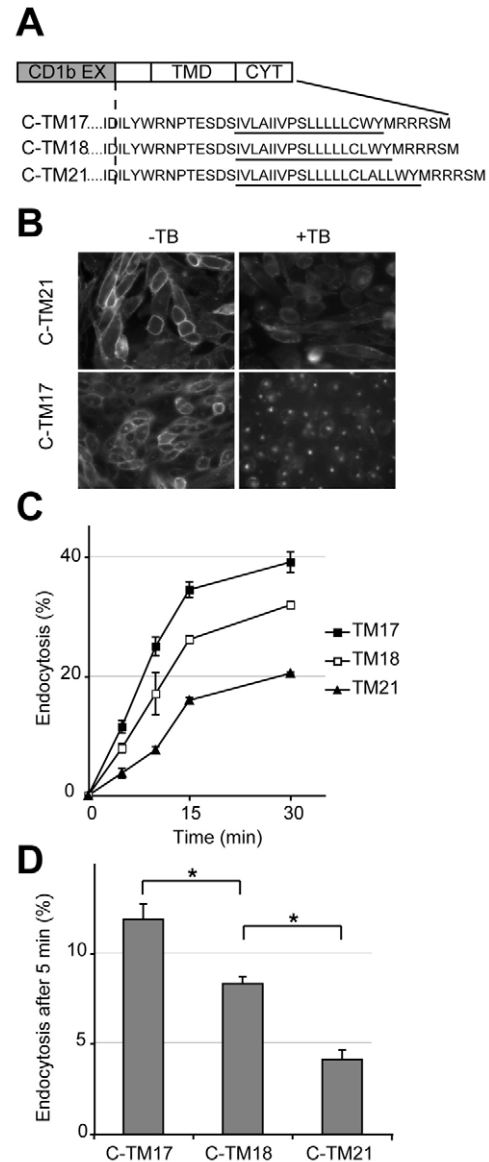
To test the influence of TMDs on transport in the endosomal pathway, we constructed and stably expressed in Chinese Hamster Ovary (CHO) cells a series of chimeric proteins comprising the

extracellular domain of CD1b fused to the TMD of various membrane proteins. We used CD1b as a reporter protein because this protein normally traffics through endocytic compartments (Jackman et al., 1998) and thus should not be sensitive to proteases found in endosomal compartments. Indeed, we have observed that both CD1b and its specific monoclonal antibody are extremely resistant to acidic pH and to proteases (data not shown). In addition, the use of a single-chain construct where  $\beta$ 2-microglobulin is fused to the CD1b heavy chain ensured that assembly of CD1b was independent of endogenous  $\beta$ 2-microglobulin (Im et al., 2004).

In mammalian cells, TMDs were shown previously to be responsible for targeting of membrane proteins to the endoplasmic reticulum (Bonifacino et al., 1991) or to the Golgi apparatus (Machamer et al., 1993; Munro, 1991). In both cases, the length of the TMD was an important parameter for determining intracellular targeting (Lankford et al., 1993; Munro, 1995). It is, however, difficult to compare the length of different TMDs, as their boundaries with the luminal and cytosolic domains are not easily defined and their amino acid compositions differ. In order to determine the effect of TMDs of different length on sorting in the endocytic pathway, we constructed a series of chimeric proteins where the length of TMDs could be directly compared. In these constructs, the TMD is delimited by positively charged residues on the cytosolic face and by negatively charged residues on the extracellular side, a common configuration in type I transmembrane proteins (Landolt-Marticorena et al., 1993). The C-TM21 construct exhibited a TMD of approximately 21 residues, and the TMD of C-TM17 was four residues shorter (17 residues; Fig. 1A). In C-TM18, the length of the TMD was 18 residues.

In agreement with previously published results (Yang et al., 1997), proteins with short TMDs (C-TM17 and C-TM18) were largely localized in the endoplasmic reticulum (ER), whereas CD1b with a long TMD (C-TM21) was mostly present at the cell surface (data not shown). Nevertheless, all of these proteins were readily detected at the cell surface (see Fig. 1B; data not shown). To assess their ability to enter endosomal compartments, we labeled surface CD1b with a green fluorescent monoclonal antibody at 4°C, then incubated the cells at 37°C for 30 minutes before quenching the surface staining with Trypan Blue (TB) to detect endocytosed CD1b (Dupont et al., 2007). This protocol might potentially induce dimerization of CD1b but it proved much more sensitive than other methods (e.g. surface biotinylation) to measure endocytosis in cells expressing low levels of surface CD1b. Whereas C-TM21 was hardly detected inside cells after incubation at 37°C, C-TM17 accumulated in a single centrally located compartment (Fig. 1B). The amount of endocytosed CD1b was quantified by flow cytometry (Fig. 1C), confirming that surface C-TM17 is endocytosed much more efficiently than the C-TM21. The internal signal accumulated slowly and reached a maximum after 15 to 30 minutes. The C-TM18 protein was also endocytosed, albeit less efficiently than C-TM17 (Fig. 1C). Even after 5 minutes, endocytosis of these three proteins was significantly different (Fig. 1D), suggesting that their rates of uptake were different.

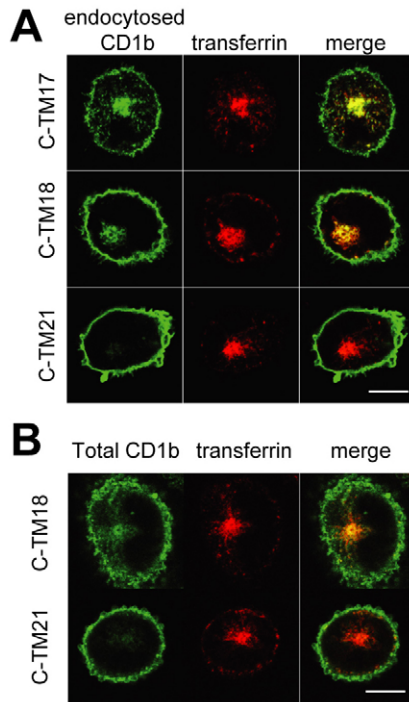
Endocytosed C-TM18 and C-TM17 were detected in a central, juxtannuclear compartment, reminiscent of recycling endosomes. To confirm this, cells were allowed to endocytose CD1b for 30 minutes in the presence of Alexa546-coupled transferrin (red), which binds to its receptor and is delivered to early and recycling endosomes. After fixation, the endocytosed CD1b was revealed with a secondary fluorescent anti-mouse antibody (green). Endocytosed C-TM18 and C-TM17 were exclusively localized in



**Fig. 1. Transmembrane domains influence protein internalization.**

(A) Chimeric CD1b proteins with transmembrane domains (TMDs) of approximately 17 (C-TM17), 18 (C-TM18) and 21 (C-TM21) amino acid residues (underlined) were stably expressed in CHO cells. (B) To assess endocytosis, cells expressing CD1b proteins were incubated at 4°C with a green fluorescent antibody directed to the CD1b extracellular domain. Cells were then incubated for 30 minutes at 37°C and the fluorescence remaining at the cell surface was quenched by addition of Trypan Blue (TB) in the medium. Low-magnification pictures reveal that C-TM17 accumulates in one centrally located endocytic compartment, whereas C-TM21 does not. (C) The amount of internalized CD1b at different times was quantified by flow cytometry. The average and s.e.m. of three independent experiments are shown. Proteins with short TMDs (C-TM18 and C-TM17) were more efficiently internalized than a protein with a long TMD (C-TM21). Endocytosis appeared complete after 30 minutes. (D) Endocytosis at 5 minutes was measured in three independent experiments for each construct. The average and s.e.m. are indicated. \* $P < 0.05$  (Mann-Whitney test).

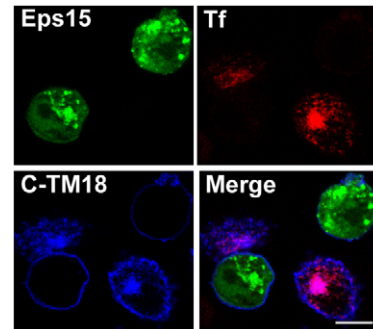
transferrin-positive recycling endosomes (Fig. 2A). This compartment was also stained with an antibody to rab11, a typical marker of recycling endosomes (Ullrich et al., 1996)



**Fig. 2. Transmembrane domains influence access to early endocytic compartments.** (A) Cells expressing CD1b proteins were incubated at 4°C with an antibody directed to the CD1b extracellular domain and with transferrin coupled to Alexa 546 (red). Cells were then incubated for 30 minutes at 37°C, fixed and the localization of the primary antibody revealed with a fluorescent secondary antibody (green). C-TM17 and C-TM18 were internalized in transferrin-positive endosomes, whereas C-TM21 was not. Representative pictures are shown. In all cells, virtually all the endocytosed CD1b was found in transferrin-positive endosomes (data not shown). Scale bar: 8  $\mu$ m. (B) In order to analyze the steady state distribution of CD1b chimeras, cells were allowed to internalize transferrin, were fixed and permeabilized, and the steady-state distribution of CD1b was revealed by immunofluorescence. C-TM18 was readily detected in transferrin-positive endosomes, whereas C-TM21 was restricted to the cell surface. Scale bar: 8  $\mu$ m.

(supplementary material Fig. S1). We also analyzed the steady-state distribution of CD1b chimeras by immunofluorescence in fixed cells, and observed that C-TM18 was present in transferrin-positive endosomes, whereas C-TM21 was not (Fig. 2B), confirming that proteins with long TMDs are restricted to the cell surface, whereas proteins with short TMDs have access to endosomal compartments.

Our interpretations concerning the role of TMDs might be erroneous if some of the proteins expressed received post-translational modifications that influenced their intracellular transport. In particular, ubiquitinylation of lysine residues, palmitoylation of cysteine residues, or direct coupling of the CD1b extracellular domain to a glycosylphosphatidylinositol (GPI) anchor could potentially affect intracellular transport. In order to formally exclude these possibilities, first we designed only chimeric CD1b proteins devoid of lysine residues in their cytosolic domain. Second, we verified that mutating the cysteine residue to alanine in the TMD did not affect sorting of the C-TM21 and C-TM17 in the endocytic pathway (supplementary material Fig. S2). Third, we verified that the sorting of C-TM21 and C-TM17 was similar in mutant CHO cells (CHO-LA1) incapable of GPI anchoring

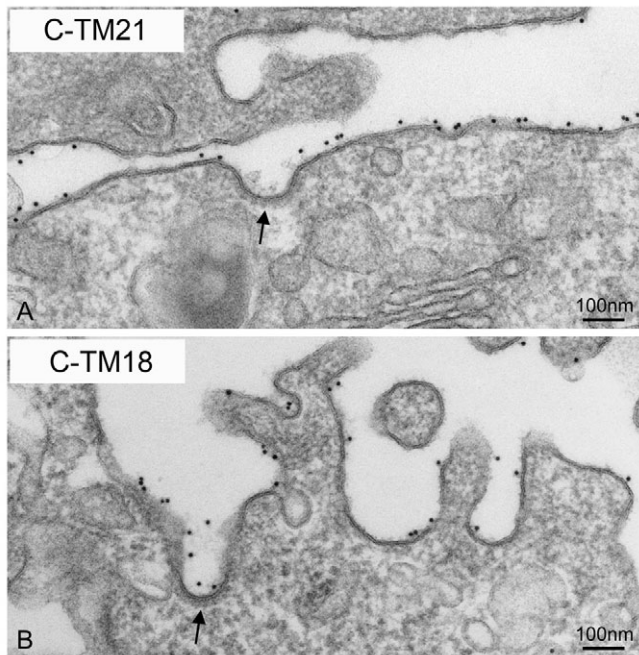


**Fig. 3. Endocytosis of C-TM18 is clathrin-dependent.** Cells expressing C-TM18 were transiently transfected with a dominant-negative mutant Eps15 fused to the green fluorescent protein (Eps15; green) to inhibit selectively clathrin-dependent endocytosis. Cells were then allowed to internalize transferrin (Tf, red) and C-TM18, were fixed and permeabilized, and the distribution of CD1b was revealed by immunofluorescence (C-TM18, blue). Endocytosis of C-TM18 and of transferrin was inhibited in cells expressing mutant Eps15. Scale bar: 8  $\mu$ m.

(supplementary material Fig. S3). Finally, we also constructed an alternative C-TM17 fusion protein, where four other amino acids were deleted from the C-TM21 protein (final sequence: DSIVLAIHVPSLLLALLWYMRR). The endocytosis of the protein was identical to that of C-TM17 (data not shown), suggesting that length of the TMD is an important determinant of intracellular sorting. It is, however, probable that other features (e.g. the exact sequence of the TMD and neighboring regions) also play a role.

Together, these results indicate that TMDs can control access of proteins to the endocytic pathway. We next investigated whether proteins with short TMDs are specifically targeted for endocytosis by clathrin-coated vesicles. To inhibit specifically the clathrin-dependent endocytic pathway, we made use of a dominant-negative mutant of Eps15 fused to GFP. This mutant protein binds to the plasma membrane adaptor AP2 and specifically inhibits clathrin-mediated endocytosis (Benmerah et al., 1999). In cells expressing mutant Eps15, we observed that endocytosis of both transferrin and C-TM18 was inhibited (Fig. 3), demonstrating that C-TM18 is internalized in clathrin-coated vesicles. To test directly and more quantitatively the influence of TMDs on endocytosis, we determined by electron microscopy the incorporation of C-TM21 and C-TM18 in clathrin-coated endocytic vesicles. These two proteins are expressed at relatively high levels at the cell surface, allowing reliable quantitative ultrastructural analysis. We fixed cells expressing either C-TM21 or C-TM18, labeled the surface CD1b protein with a specific antibody and gold-coupled protein A, and observed the samples by electron microscopy. We then quantified the amount of labeling inside and outside of clathrin-coated pits. C-TM21 was virtually absent from clathrin-coated pits (0.46 gold/ $\mu$ m in clathrin-coated pits versus 4.1 gold/ $\mu$ m in uncoated plasma membrane), whereas C-TM18 was present, albeit at a slightly lower concentration than observed at the rest of the cell surface (1.13 gold/ $\mu$ m in clathrin-coated pits versus 1.7 gold/ $\mu$ m in uncoated plasma membrane) (Fig. 4; Table 1). Together, these results suggest that TMDs influence endocytosis by controlling the degree of exclusion of transmembrane proteins from clathrin-coated pits and vesicles.

To test if these observations were also applicable to the TMDs of other proteins, we constructed and expressed in CHO cells a



**Fig. 4. Transmembrane domains control exclusion from clathrin-coated pits.** Cells expressing C-TM21 (A) or C-TM18 (B) were fixed and surface CD1b was labeled with gold particles. The samples were then processed and analyzed by electron microscopy. C-TM18 was present in clathrin-coated pits (arrows), whereas C-TM21 was virtually excluded. Scale bar: 100 nm. A quantitative analysis is presented in Table 1.

series of chimeric proteins comprising the extracellular domain of CD1b fused to the TMD of various membrane proteins (CD1a, CD1b, CD4 and MHC-class I). The corresponding constructs were named C-CD1a, C-CD1b, C-CD4 and C-MHCI, respectively. As the boundary between the extracellular and transmembrane domains is often not clearly defined, we also included a small segment of the extracellular juxtamembrane region of these proteins in the chimeric proteins and a minimal cytosolic domain (Fig. 5A). We assessed the ability of these chimeric proteins to enter endosomal compartments as described above. After 30 minutes of internalization, endocytosed C-CD1a was clearly detected in transferrin-positive endosomes (Fig. 5B). On the contrary, C-MHCI was not efficiently endocytosed (Fig. 5B). To quantify these results, we determined the fluorescence of endocytosed CD1b in transferrin-positive endosomes and compared it with the signal remaining at the plasma membrane (Fig. 5C). Endocytosis of all chimeric proteins was also assessed by flow cytometry, as described above,

yielding results very similar to those presented below but with slightly more variability (supplementary material Fig. S4). The results of four independent experiments confirmed that chimeric CD1b proteins with different TMDs entered cells at very different rates. Some TMDs (CD1a) dictated efficient endocytosis, others a more modest endocytosis (CD1b), and others essentially restricted chimeric proteins to the cell surface (MHCI, CD4; Fig. 5D; supplementary material Fig. S4A). These results indicate that, in the absence of a cytosolic endocytic signal, access of a membrane protein to endosomal compartments is controlled at least in part by its TMD. Some TMDs allow surface proteins to reach endosomal compartments, others are more restricted to the cell surface.

If both the transmembrane and the cytosolic domains of a protein can control incorporation into clathrin-coated pits, what happens if transmembrane and cytosolic determinants dictate different fates? We investigated the fate of a protein where the TMD would not allow endocytosis but the cytosolic domain would contain an endocytic signal. For this, we added to the C-TM21 protein (normally restricted to the cell surface) the full-length cytosolic domain of CD1b, which has been shown previously to cause endocytosis of the protein owing to the presence of a canonical YxxL motif (Jackman et al., 1998) (Fig. 6A). We then assessed the ability of these chimeric proteins to be internalized as described above. The C-TM21-YxxL chimeric protein was internalized more efficiently than C-TM21 (Fig. 6B; supplementary material Fig. S4B), indicating that the addition of a cytosolic endocytosis signal increased the efficiency of endocytosis of C-TM21. Remarkably, the level of endocytosis observed remained lower than that of C-TM17 (Fig. 6B; supplementary material Fig. S4B), indicating that endocytosis mediated by a TMD can in some cases be more efficient than endocytosis mediated by a well-characterized cytosolic sorting motif. The C-TM18-YxxL protein, where the endocytosis signal was added to a protein with a short TMD (Fig. 6A), was more efficiently endocytosed than C-TM21-YxxL (Fig. 6B; supplementary material Fig. S4B), further demonstrating the importance of TMDs in determining endocytosis. These results indicate that the combined action of the transmembrane and cytosolic domains determines the efficacy with which a membrane protein is endocytosed.

## Discussion

The textbook view of endocytosis proposes that cytosolic endocytic signals cause endocytosis of membrane proteins by driving their active concentration in clathrin-coated pits owing to specific interactions with clathrin adaptor complexes. Our observations add three new notions to this classical model. First, our results indicate that in the absence of cytosolic sorting signals, TMDs act as sorting

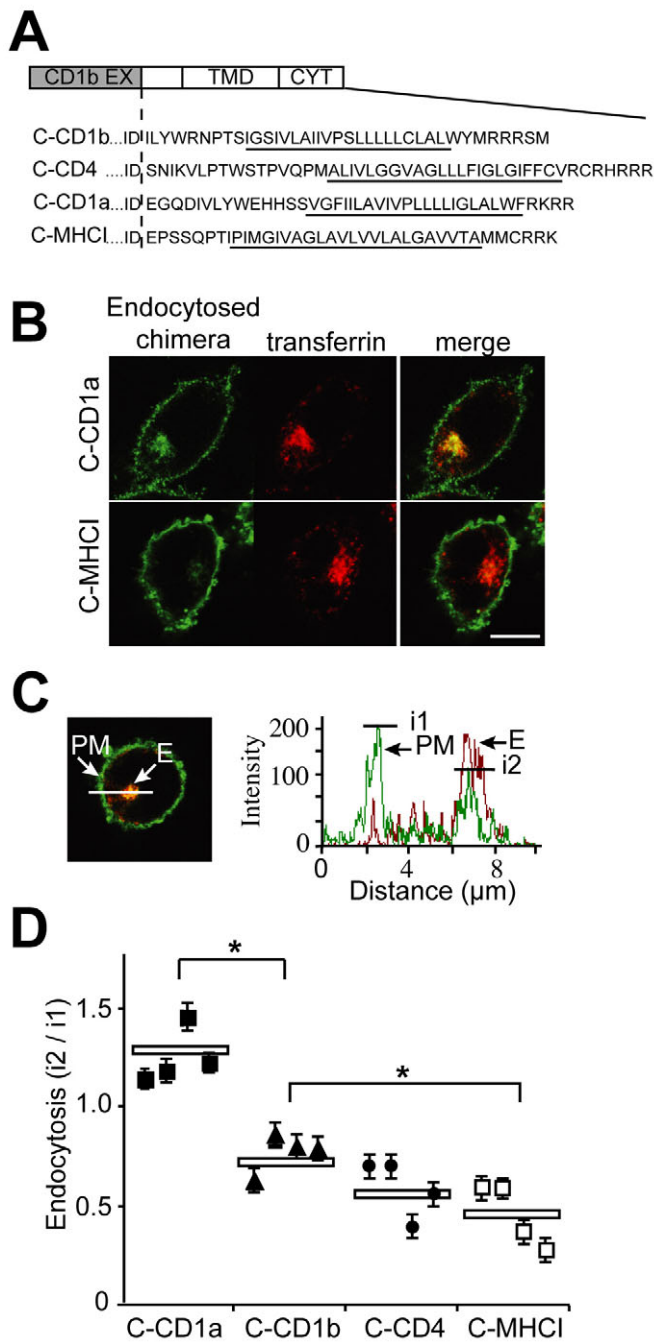
**Table 1. Localization of CD1b chimeric proteins in clathrin-coated pits**

|        | Exp   | Surface clathrin-coated pits (CCP) |                          |                |                     | Uncoated plasma membrane (PM) |                |                     | CCP/PM ratio |
|--------|-------|------------------------------------|--------------------------|----------------|---------------------|-------------------------------|----------------|---------------------|--------------|
|        |       | Number                             | Length ( $\mu\text{m}$ ) | Gold particles | Gold/ $\mu\text{m}$ | Length ( $\mu\text{m}$ )      | Gold particles | Gold/ $\mu\text{m}$ |              |
| C-TM21 | 1     | 27                                 | 5.686                    | 0              | 0.00                | 21.816                        | 42             | 1.92                | 0            |
|        | 2     | 33                                 | 7.769                    | 2              | 0.25                | 26.888                        | 96             | 3.57                | 0.07         |
|        | 3     | 19                                 | 4.415                    | 5              | 1.13                | 12.536                        | 85             | 6.78                | 0.16         |
|        | 4     | 13                                 | 3.821                    | 3              | 0.78                | 7.231                         | 60             | 8.29                | 0.09         |
|        | Total | 92                                 | 21.573                   | 10             | 0.46                | 68.471                        | 283            | 4.1                 | 0.11         |
| C-TM18 | 1     | 37                                 | 8.774                    | 8              | 0.91                | 34.039                        | 50             | 1.46                | 0.62         |
|        | 2     | 33                                 | 8.145                    | 13             | 1.59                | 23.833                        | 54             | 2.26                | 0.70         |
|        | 3     | 10                                 | 2.666                    | 5              | 1.87                | 6.910                         | 15             | 2.17                | 0.86         |
|        | 5     | 33                                 | 7.826                    | 5              | 0.64                | 38.076                        | 57             | 1.50                | 0.42         |
|        | Total | 113                                | 27.411                   | 31             | 1.13                | 102.858                       | 176            | 1.71                | 0.66         |

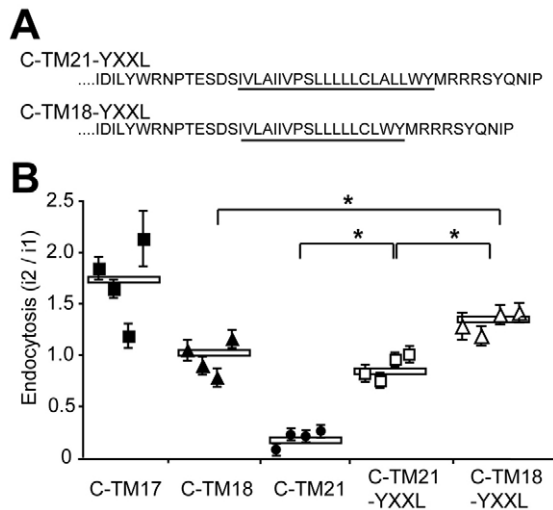
determinants during endocytosis. Accordingly, two proteins devoid of cytosolic endocytic signals (e.g. C-TM21 and C-TM18) can be endocytosed differently depending on their TMDs. Second, our results suggest that TMDs influence endocytosis by controlling exclusion of transmembrane proteins from clathrin-coated pits. We notably observed that a reporter protein with a long TMD (C-TM21) was virtually absent from vesicles in formation, whereas the same protein with a shorter TMD (C-TM18) was present in clathrin-coated pits, albeit at a slightly lower concentration than at the rest of the cell surface. Although efficient endocytosis clearly relies on an active concentration of proteins in clathrin-coated pits, for proteins that are less efficiently endocytosed it is the degree of exclusion from clathrin-coated vesicles that is the crucial element.

Thus, during the formation of clathrin-coated vesicles, the degree of incorporation of various membrane proteins can cover a large spectrum from nearly total exclusion to efficient concentration. Third, we observed that the combination of sorting information in transmembrane and cytosolic domains determines the efficiency with which a given membrane protein is internalized. In some cases, internalization driven by a TMD can be more efficient than that driven by a cytosolic endocytic motif (for example C-TM17 versus C-TM21-YxxL). When studying the traffic of a membrane protein, TMD-dependent sorting might prove as crucial as cytosolic sorting signals to determine the efficacy of endocytosis.

There are severe technical limitations to the observation of membrane exclusion in intracellular transport vesicles, explaining in part why membrane exclusion has been largely ignored so far. Only electron microscopy provides the resolution necessary to observe unambiguously intracellular transport vesicles and, even with this technique, vesicles in the process of budding can be difficult to identify. In addition, intracellular membrane compartments are not accessible to labeling prior to sectioning and this imposes labeling to be performed on thin cryosections, a particularly demanding technique. Here, we have been able to assess exclusion from clathrin-coated pits because the plasma membrane is readily accessible to pre-embedding labeling and because clathrin-coated pits are easily identified at the ultrastructural level. It is, however, probable that selective exclusion of membrane proteins occurs at every step of intracellular transport. During an earlier analysis of intra-Golgi transport, we indeed observed that resident Golgi proteins are less abundant at sites where vesicles form (the rims of cisternae), suggesting at least partial exclusion from transport vesicles (Cosson et al., 2002; Cosson et al., 2005; Orci et al., 2000). Similarly, we reported selective membrane exclusion during the formation of phagocytic or macropinocytic cups in *Dictyostelium* cells (Mercanti et al., 2006). More recently, a study showed that proteins with short TMDs are excluded from ER exit sites, whereas proteins with longer TMDs are concentrated at these sites (Ronchi et al., 2008). Our observations suggest that the reverse is true at the cell surface: proteins with long TMDs are



**Fig. 5. Transmembrane domains influence access of surface proteins to endocytic compartments.** (A) Plasmids encoding various chimeric proteins were constructed and stably transfected in CHO cells. Chimeric proteins comprised the extracellular domain of CD1b (EX, gray box) fused to the membrane-proximal, the TMD and a minimal cytosolic domain (CYT) of various membrane proteins. The putative TMDs are underlined. (B) Endocytosis of chimeras was assessed as described in Fig. 2A. After 30 minutes of internalization, endocytosed C-CD1a was clearly detected in transferrin-positive endosomes, whereas C-MHCI did not accumulate efficiently in endocytic compartments. Scale bar: 8  $\mu\text{m}$ . (C) To quantify the amount of endocytosed CD1b protein in transferrin-positive endosomes, images were analyzed with line scan (white line), as shown in the left panel. In the right panel, the line scan reflects the intensity of CD1b staining (green line) and of transferrin staining (red line). The first peak (i1) corresponds to CD1b still present at the plasma membrane (PM), and the second peak (i2) to CD1b endocytosed in transferrin-positive endosomes (E). The i2/i1 ratio measures the accumulation of endocytosed material in endosomes. (D) For each chimeric protein, four independent experiments were performed and quantified. For each experiment, 20 images were quantified, the average and s.e.m. are indicated. The white bar shows the average of the four experiments. Chimeric proteins accumulated at different rates, revealing the influence of TMDs on membrane sorting in the endocytic pathway. Global statistical analysis (Kruskal-Wallis test) indicated the existence of significant differences ( $P < 0.05$ ). \* $P < 0.05$  (Mann-Whitney test).

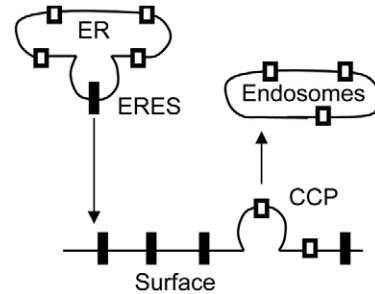


**Fig. 6. The combined action of the TMD and cytosolic domain determines the efficacy of endocytosis.** (A) To investigate the relative influence of TMDs and cytosolic domains in controlling endocytosis, we added to C-TM21 or to C-TM18 the full-length cytosolic domain of CD1b containing a YxxL endocytic signal. (B) Endocytosis of chimeras was assessed as described in Fig. 2A and quantified as described in Fig. 5C. C-TM21-YxxL (respectively, C-TM18-YxxL) was internalized more efficiently than C-TM21 (respectively, C-TM18), indicating that the addition of a cytosolic endocytosis signal increased the efficiency of endocytosis. The level of endocytosis of C-TM21-YxxL remained lower than that of C-TM17, indicating that endocytosis mediated by a TMD can in some cases be more efficient than endocytosis mediated by a well-characterized cytosolic sorting motif. The C-TM18-YxxL protein was more efficiently endocytosed than C-TM21-YxxL, further demonstrating the importance of TMDs in determining endocytosis. \* $P < 0.05$  (Mann-Whitney test).

preferentially excluded from endocytic vesicles at that stage. These sorting mechanisms might cooperate to ensure efficient exclusion of proteins with short TMDs from the cell surface. A simplified view of these results is proposed (Fig. 7).

It should be stressed that our results do not establish that the length of a TMD is the only element determining its effect on sorting. It is indeed virtually impossible to make a statement on this point, as the exact lengths of two different TMDs cannot be precisely compared if they were not designed for that purpose (for example, C-TM17, C-TM18 and C-TM21). It is even difficult to compare the length of two very similar TMDs like C-TM21 and C-CD1b as there are no clear rules today to define the exact boundaries of each TMD. The exact composition of a TMD, in addition to its length, is most probably an important determinant of its effect on sorting.

One of the aims of cellular biology is to determine how various sorting signals cooperate to ensure the precise targeting of each protein in exocytic and endocytic compartments. Whereas a lot of attention has been focused on identifying cytosolic sorting signals and cytosolic coats, TMDs might also be essential sorting determinants controlling exclusion of transmembrane proteins from transport vesicles. Further work will be necessary to characterize membrane exclusion at various stages of intracellular transport and to identify more extensively the features that determine exclusion of membrane proteins. The molecular mechanisms that ensure recognition and sorting of TMDs are essentially unknown, and also constitute a fascinating field for future investigations.



**Fig. 7. A simplified view of TMD sorting in the ER and at the cell surface.** Proteins with short TMDs (empty rectangles) are excluded from ER exit sites (ERES) (Ronchi et al., 2008), whereas they are preferentially included in clathrin-coated pits (CCP) (this study). On the contrary, proteins with long TMDs (black rectangles) are included in ERES and excluded from CCPs. Thus, sorting of TMDs in the ER and at the cell surface cooperate to ensure exclusion of proteins with short TMDs from the cell surface. This simplified figure does not take into account the effect of cytosolic sorting signals on intracellular transport both in the endocytic and in the exocytic pathways. Although for simplicity TMDs are referred to here simply as long or short, it is probable that their exact composition is also crucial in determining their effect on intracellular sorting.

## Materials and Methods

### Cells and reagents

CHO cells were grown at 37°C in a mixture of F-12 medium and Dulbecco's Modified Eagle's Medium (DMEM-F12, Invitrogen) supplemented with GlutaMax-1, Pyridoxine and 10% Fetal Calf Serum (Invitrogen). CHO-LA1 cells defective for GPI anchoring (Abrami et al., 2001) were a kind gift of Dr G. van der Goot (Ecole Polytechnique Fédérale de Lausanne, Switzerland). For immunofluorescence, a mouse monoclonal antibody to the extracellular domain of CD1b was used (4A7) (Olive et al., 1984). A rabbit polyclonal antiserum to rab11 (A2) was a kind gift of S. Lodeho (Institut Curie, Paris, France). To label endosomes, transferrin from human serum coupled to Alexa 546 (Molecular Probes) was used. When indicated, the mouse monoclonal 4A7 was coupled with Alexa 488 fluorochrome (Molecular Probes).

The vector coding for a dominant-negative mutant Eps15 (EΔ95/295) (Benmerah et al., 1999) was obtained from Dr G. van der Goot. The cDNA coding for the human single chain CD1b was a kind gift of S. Porcelli (Im et al., 2004). It was subcloned into the pRC-RSV expression plasmid using polymerase chain reaction (PCR). A *Clal* site was introduced on the luminal side of the TMD and an *XbaI* site after the Stop codon. All mutants were constructed by annealing complementary oligonucleotides and subcloning them into the *Clal* and *XbaI* sites. For transfection, CHO cells were plated on 100 mm culture dishes and transformed with 20 μg of the plasmid DNA using the calcium phosphate precipitation method (Graham and van der Eb, 1973). Stable clones were isolated after Geneticin selection (700 μg/ml). Transfected cells were grown in the presence of G418 and transferred to a G418-free medium two days before usage.

### Immunofluorescence microscopy

To visualize CD1b endocytosis, stable clones expressing CD1b chimeric proteins were grown on 20 mm glass coverslips for 2 days. To assess CD1b endocytosis, the cells were rinsed and incubated for 10 minutes at 4°C in DMEM-F12 containing the Alexa 488-coupled 4A7 antibody, washed twice with medium and incubated at 37°C in medium for different lengths of time. Cells were then incubated in medium with or without Trypan Blue (freshly made, 8 mg/ml). Live cells were then directly observed with an epifluorescence microscope (Leica, Wetlar, Germany).

To determine access of CD1b in recycling endosomes, cells were incubated at 4°C for 10 minutes in DMEM-F12 medium (without FCS) containing the 4A7 anti-CD1b antibody and Alexa 546-transferrin (50 μg/ml), washed twice with medium and incubated at 37°C in medium containing Alexa 546-transferrin for 0 or 30 minutes. Cells were then fixed for 30 minutes with 4% paraformaldehyde, permeabilized with 0.2% Saponin for 10 minutes, incubated for 30 minutes with the Alexa 488 secondary antibody against mouse IgG (Molecular Probes) and coverslips were mounted in Moewiol (Heimer and Taylor, 1974) and observed with an LSM510 confocal microscope (Carl Zeiss, Feldbach, Switzerland).

For quantitative analysis of the pictures, the 'Profile' tool of the LSM510 confocal microscope software was used. For each fluorescence image, a line was drawn through the plasma membrane and transferrin-positive endosomes (Fig. 5C, left panel, white line). The fluorescence intensity in endosomes (i2) was divided by the

fluorescence intensity of the cell surface (i1) after subtraction of the background fluorescence (measured inside the cells). In each experiment, 20 cells were analyzed and the average and s.e.m. are indicated.

#### Flow cytometry

Stable clones expressing CD1b chimeric proteins were allowed to detach in PBS containing 0.5 mM EDTA, incubated for 10 minutes at 4°C in medium containing Alexa 488-coupled 4A7, then rinsed and incubated at 37°C in medium for 0, 5, 10, 15 or 30 minutes. Cells were then incubated in PBS with or without Trypan Blue dye (freshly made, 8 mg/ml) for 5 minutes before analysis with a flow cytometer (FACScalibur). Cells were identified based on their forward and side scatter. For quantitative analysis, at each time we calculated the percentage of CD1b staining that was not quenched by Trypan Blue.

#### Electron microscopy

To evaluate the presence of CD1b in clathrin-coated pits, cells expressing either C-TM21 or C-TM18 were plated 2 days before the experiment, fixed for 15 minutes in medium containing 4% paraformaldehyde and for 15 minutes in 0.1 M phosphate buffer pH 7.4 containing 4% paraformaldehyde. The cells were then incubated for 30 minutes in PBS containing 0.2% BSA (PBS/BSA) and the anti-CD1b antibody, washed and incubated for 30 minutes with rabbit IgG anti-mouse immunoglobulin (6.2 µg/ml in PBS/BSA). Cells were then incubated for 30 minutes with 10 nm gold-coupled protein A (Cell Microscopy Center, Utrecht, The Netherlands) diluted 1:10 in PBS/BSA, rinsed and fixed for 30 minutes in 0.1 M phosphate buffer pH 7.4 containing 2% glutaraldehyde. Fixed cells were dehydrated, embedded in Epon resin and processed for conventional electron microscopy as described previously (Orci et al., 1973). Grids were examined using a Tecnai transmission electron microscope (FEI, Eindhoven, The Netherlands). For quantitative analysis of images, Tecnai Analysis software was used. For each experiment, we determined the density of gold particles in surface clathrin-coated pits and in uncoated regions of the plasma membrane and calculated the ratio of these two values to determine the relative density of labeling in coated regions of the membrane. For each construct, the results of four independent experiments are shown in Table 1.

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Supplementary material available online at

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