Identification of a dominant endoplasmic reticulum-retention signal in yellow fever virus pre-membrane protein

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Yellow fever virus (YFV) encodes two envelope proteins, pre-membrane (prM) and envelope (E), that accumulate in the endoplasmic reticulum (ER). The C termini of prM and E form two antiparallel transmembrane α-helices that contain ER-retention signals. To understand further the ER retention of the prME heterodimer, we characterized the subcellular localization of chimeric proteins made of a reporter protein fused to the transmembrane segments of YFV envelope proteins. We showed that at least three of the transmembrane segments of the prME heterodimer are ER-retention signals. Interestingly, increasing the length of these α-helices led to the export of the chimeric proteins out of the ER. Furthermore, adding a diacidic export signal at the C terminus of the first transmembrane segment of the E protein also induced export to the cell surface. However, adding this export signal at the C terminus of the first transmembrane segment of E in the context of prME did not change the subcellular localization of the prME heterodimer, suggesting the presence of a stronger ER-retention signal outside the first transmembrane segment of E. Importantly, the diacidic export motif added to the C terminus of the first transmembrane segment of the prM protein was not sufficient to export a chimeric protein out of the ER, indicating that this sequence is a dominant ER-retention signal. Together, these data indicate that a combination of several signals of different strengths contributes to the ER retention of the YFV envelope protein heterodimer.

INTRODUCTION

Yellow fever virus (YFV) belongs to the genus Flavivirus in the family Flaviviridae. Flaviviruses are small, enveloped, positive-stranded RNA viruses. The flavivirus particle is made of an envelope, containing the envelope (E) and membrane (M) proteins, that surrounds a nucleocapsid composed of genomic RNA and multiple copies of the capsid protein (Kuhn et al., 2002). The M protein is synthesized as a precursor called the pre-membrane (prM) protein that associates with E in the endoplasmic reticulum (ER) to form heterodimers (Allison et al., 1995; Wengler, 1989). The prM and E envelope proteins contain a large ectodomain and a C-terminal membrane anchor (Fig. 1a). Interestingly, in YFV-infected cells, the prME heterodimer accumulates in the ER (Op De Beeck et al., 2004). This subcellular localization is likely to be essential for virion morphogenesis, which occurs in association with ER membranes (reviewed by Lindenbach et al., 2007).

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Fig. 1. The lack of an export signal contributes to the ER retention of CD4–TM1E. (a) Topology of YFV envelope proteins, prM and E contain a large luminal domain and a C-terminal membrane anchor. The C termini of these proteins form two antiparallel transmembrane α-helices (TM1 and TM2). A sequence alignment of the transmembrane regions of YFV, dengue virus 1 (DEN1), West Nile virus (WNV) and Japanese encephalitis virus (JEV) is shown. The putative minimal transmembrane sequences (Cocquerel et al., 2000) are indicated by #. (b) Schematic diagrams of CD4 and chimeras containing the CD4 ectodomain fused to wild-type or mutated TM1 of E containing or not the cytosolic tail of vesicular stomatitis virus glycoprotein (VSV-G), mutated (CD4–TM1E–Gmut) or not (CD4–TM1E–G) in the D1E motif. The sequences of wild-type and mutated TM1 of E are indicated below the diagrams and the putative minimal transmembrane sequence is highlighted in grey. The mutated TM1 of E [TM1E(Ala)3] had a stretch of hydrophobic residues (ALALAL sequence) added in the middle to increase the size of TM1. cyto, Cytosolic domain; SP, signal peptide; TM, transmembrane segment. (c) Immunolocalization of wild-type and chimeric CD4 proteins. HeLa cells transfected with the appropriate plasmids were processed for immunofluorescence detection of CD4. Representative images of cells expressing CD4, CD4–TM1E, CD4–TM1E(Ala)3, CD4–TM1E–G and CD4–TM1E–Gmut are shown. (d) Detection of cell surface-biotinylated proteins. After biotinylation, the recombinant proteins were pulled down (biotin) or not (lysate) with streptavidin–agarose beads, separated by SDS-PAGE and revealed by Western blotting with an anti-CD4 antibody. A molecular mass marker is indicated on the left.
of a reporter protein fused to the transmembrane sequences of YFV envelope proteins. Interestingly, we identified several ER-retention signals in the transmembrane regions. We showed that the length of these transmembrane segments can play a role in ER retention. Furthermore, we also showed that a balance of forces between ER-retention and export signals can lead to different outcomes depending on the strength of the ER-retention signals. Indeed, export to the plasma membrane was observed when a diacidic export motif was fused at the C terminus of the first transmembrane segment of the E protein, but not when the same signal was added at the C terminus of the first transmembrane segment of the prM protein.

RESULTS

Increasing the length or adding a diacidic export motif suppresses ER retention mediated by TM1 of E

We have shown previously that the first transmembrane segment of YFV E protein (TM1) contains an ER-retention signal (Op De Beeck et al., 2004). As it is known that increasing the length of transmembrane domains can disrupt ER retention of some proteins (Pedrazzini et al., 1996; Szczesna-Skorupa & Kemper, 2000; Yang et al., 1997), we tested whether adding two helix turns in TM1 of E would lead to a different subcellular location of a chimeric protein containing the ectodomain of CD4 in fusion with this transmembrane segment (CD4–TM1E) (Fig. 1b). Indeed, as prM plays a chaperone role for the folding of E (Konishi & Mason, 1993), replacing the ectodomain of E was necessary to avoid misfolding of this protein due to the absence of prM, which would lead indirectly to ER retention. To increase the size of TM1 of E, we added a stretch of hydrophobic residues in the middle of TM1 of E and compared the subcellular localization of this protein (CD4–TM1E(AL)3) with that of CD4–TM1E and wild-type CD4. The ALALAL sequence was chosen, as both alanine and leucine residues are observed frequently in transmembrane domains. Interestingly, this insertion led to export of the chimeric protein at the plasma membrane (Fig. 1c). Indeed, as for wild-type CD4, the immunofluorescent signal was detected predominantly at the plasma membrane when the VSV-G cytosolic tail was added at the C terminus of CD4–TM1E (CD4–TM1E–G), whereas the CD4–TM1E molecule was retained in the ER. Furthermore, mutation of the export motif within the VSV-G tail led to an intracellular localization of the chimera (CD4–TM1E–Gmut) very similar to that of CD4–TM1E. The export of CD4–TM1E–G was confirmed by analysing cell surface-biotinylated proteins. In contrast to CD4–TM1E or CD4–TM1E–Gmut, a large fraction of CD4–TM1E–G was detected at the plasma membrane, similar to what was observed for wild-type CD4 (Fig. 1d). A quantitative analysis indicated that CD4–TM1E–G was exported to the plasma membrane at the same level as the control CD4 protein, whereas only 5% of CD4–TM1E or TM1E–Gmut was exported to the cell surface. Together, these data indicate that the lack of an export signal contributes to the ER retention of CD4–TM1E.

Tagging the prME heterodimer with a diacidic export motif does not relieve ER retention

As adding an export signal at the C terminus of CD4–TM1E led to export out of the ER, we wondered whether such a signal would also be able to induce the export of the prME heterodimer out of the ER. We therefore fused a VSV-G cytosolic tail at the C terminus of prME. Deletion of the second transmembrane segment of the E protein (TM2) was necessary to position the export motif of VSV-G into the cytosol. Such a deletion does not affect the functions of the prME heterodimer. Indeed, a similar deletion in the prME heterodimer of tick-borne encephalitis virus, another flavivirus, has been shown to maintain the functions of the envelope proteins (Allison et al., 1999). As shown in Fig. 2, adding an export motif at the C terminus of the prME heterodimer did not affect its subcellular localization. Indeed, immunofluorescence
detection of the tagged E protein with an anti-E antibody (2D12) or an antibody recognizing the VSV-G cytosolic tail (P5D4) showed an intracellular accumulation of the prME–G protein, similar to what was observed for wild-type prME. In contrast, CD4–TM1E–G, which was used as a control for export out of the ER, showed accumulation of the fluorescent signal at the plasma membrane and the Golgi apparatus. These data indicate that additional retention signal(s) in the transmembrane region of prM competes with the export signal present in the VSV-G cytosolic tail.

**Fig. 2.** The DIE diacidic export motif does not relieve the ER retention of the prME heterodimer. (a) Schematic diagrams of wild-type prME polyprotein deleted of TM2 of E and containing (prME–G) or not (prME) the cytosolic tail of VSV-G in fusion with TM1 of E. On the top of the panel is also shown the control chimeric CD4 protein containing the ectodomain of CD4 in fusion with TM1 of E and containing the cytosolic tail of VSV-G (CD4–TM1E–G). SP, Signal peptide. (b, c) Immunolocalization of wild-type or chimeric E and CD4 proteins. HeLa cells transfected with the appropriate plasmids were processed for immunofluorescence detection with either monoclonal antibody (mAb) P5D4, recognizing the VSV-G cytosolic tail (b), or mAb 2D12, recognizing the YFV E protein (c). Representative images of cells expressing CD4–TM1E–G, prME and prME–G are shown.

**The transmembrane region of prM contributes to the ER retention of prME in the presence of a diacidic motif**

The absence of export of prME–G out of the ER suggests that additional signals present in prME have a strong ER-retention capacity. To determine whether a strong ER-retention signal is located in the ectodomains or in the transmembrane regions, we constructed a fusion protein devoid of prME ectodomains and containing the ectodomain of CD4 at the N terminus and a VSV-G cytosolic tail at the C terminus (Fig. 3a; CD4–TMprM–TM1E–G), and we analysed its subcellular localization by immunofluorescence. As shown in Fig. 3(b), adding an export signal at the C terminus of a chimeric protein containing only the transmembrane regions of prME did not lead to export of these proteins out of the ER. In contrast, the control CD4–TM1E–G protein showed accumulation of the fluorescent signal at the plasma membrane and the Golgi apparatus. Furthermore, colocalization studies of CD4–TMprM–TM1E–G with the Golgi marker GM130 (Fig. 3c) and the ER marker calnexin (Fig. 3d) showed that the chimeric protein is retained in the ER. Together, these data indicate that additional retention signal(s) in the transmembrane region of prM competes with the export signal present in the VSV-G cytosolic tail.

**TM1 of prM is a dominant ER-retention signal**

The first transmembrane segment of prM (TM1) also contains an ER-retention signal (Op De Beeck et al., 2004), and we therefore wanted to characterize this signal further. As for the ER-retention signal present in TM1 of E, we tested whether adding two helix turns in TM1 of prM would lead to a different subcellular localization of a chimeric protein containing the ectodomain of CD4 in fusion with TM1 of prM (Fig. 4a). We therefore added the ALALAL sequence in the middle of TM1 of prM (CD4–TM1prM(AL)3), and we compared the subcellular localization of CD4–TM1prM(AL)3 with that of CD4–TM1prM and wild-type CD4. Interestingly, as for CD4–TM1CD4–TM1E(AL)3, this insertion led to an export of the chimeric protein at the plasma membrane (Fig. 4b). Indeed, as for the control wild-type CD4, the immunofluorescent material was detected predominantly at the plasma membrane when the ALALAL sequence was added in the transmembrane domain of CD4–TM1prM (CD4–TM1prM(AL)3), whereas the non-mutated CD4–TM1prM molecule was retained in an intracellular compartment, which has been shown previously to be the ER (Op De Beeck et al., 2004). The export of CD4–TM1prM(AL)3 was confirmed by analysing cell surface-biotinylated proteins. In contrast to CD4–TM1prM, a large fraction of CD4–TM1prM(AL)3 was detected at the plasma membrane, similar to what was observed for wild-type CD4 (Fig. 4c). A quantitative analysis indicated that the level of export of CD4–TM1prM(AL)3 at the plasma membrane was similar to that of the CD4 control protein, whereas only 9% of CD4–TM1prM was exported to the cell surface. However, in contrast to what was observed for TM1 of E, changing the localization of the ALALAL insertion in TM1 of prM led to only partial export of the chimeric CD4–TM1prM(AL)3bis protein (Fig. 4c, d). Indeed, a quantitative analysis indicated that approximately 30% of CD4–
TM1prM(AL)3bis was exported to the cell surface. These data suggest that an ER-retention motif is present in the first transmembrane segment of prM, regulating its ER-retention function.

As for the TM1 of E, we also tested whether adding an export signal at the C terminus of TM1 of prM can induce export out of the ER. This was analysed by fusing a VSV-G cytosolic tail at the C terminus of CD4–TM1E (Fig. 4a; CD4–TM1E–Gmut). In contrast to CD4–TM1E–Gmut, the presence of the DIE motif did not lead to export of the chimeric CD4–TM1prM protein to the plasma membrane (Fig. 4b). Indeed, as for the non-mutated CD4–TM1prM, the immunofluorescent signal was detected predominantly in an intracellular compartment. Furthermore, co-immunostaining with markers of the ER (calnexin) (Fig. 4b) and the Golgi (GM130) (data not shown) indicated that CD4–TM1prM–Gmut is retained in the ER. The lack of export of CD4–TM1prM–Gmut was confirmed by analysing cell surface-biotinylated proteins. In contrast to

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**Fig. 3.** The transmembrane region of prM contributes to the ER retention of prM in the presence of a diacidic motif. (a) Schematic diagrams of chimeric CD4 proteins containing the ectodomain of CD4 in fusion with TM1 of E or the transmembrane region of prM in fusion with TM1 of E and containing or not VSV-G cytosolic tail, mutated or not in the DIE motif. To add a spacer between the transmembrane domain of prM and the TM1 of E, a haemagglutinin (HA) epitope was inserted. SP, Signal peptide. (b) Immunolocalization of the chimeric CD4 proteins. HeLa cells transfected with the appropriate plasmids were processed for immunofluorescence detection of CD4. Representative images of cells expressing CD4–TM1E–G, CD4–TM1E–Gmut, CD4–TMprM–TM1E–G and CD4–TMprM(AL)3 bis–TM1E–G are shown. (c, d) Confocal immunofluorescence analysis of the localization of CD4–TMprM–TM1E–G. HeLa cells transfected with the appropriate plasmids were processed for immunofluorescence staining for the detection of CD4 [left panels in (c) and (d)], GM130 as Golgi marker or calnexin (CNX) as ER marker [middle panels in (c) and (d)]. Colocalization was visualized by overlay (right panels). Representative images of cells expressing CD4–TMprM–TM1E–G are shown.
A dominant ER-retention signal in a viral protein

Fig. 4. TM1 of prM is a dominant ER-retention signal. (a) Schematic diagrams of wild-type CD4 and chimeric proteins containing the ectodomain of CD4 in fusion with wild-type or mutated TM1 of prM and containing or not a VSV-G cytosolic tail. The sequences of wild-type and mutated TM1 of prM are indicated below the diagrams and the putative minimal transmembrane sequence of the wild type (Cocquerel et al., 2000) is highlighted in grey. The mutated TM1 of prM had the sequence ALALAL added in the middle (TM1prM (AL)₃) or to one side (TM1prM (AL)₃bis). cyto, Cytosolic domain; SP, signal peptide; TM, transmembrane segment. (b) Confocal immunofluorescence analysis of the localization of CD4–TM1prM, CD4–TM1prM (AL)₃ and CD4–TM1prM–G. HeLa cells transfected with the appropriate plasmids were processed for immunofluorescence staining for the detection of CD4-derived proteins with a specific mAb (panels labelled CD4) and with an anti-calnexin (CNX) antibody as an ER marker. Representative images of cells expressing CD4–TM1prM, CD4–TM1prM (AL)₃ and CD4–TM1prM–G are shown. Enlargements of the boxed areas are shown in the small panels to the right of the Overlay column. Bar, 10 μm. (c) Detection of cell surface-biotinylated proteins. After biotinylation, the recombinant proteins were pulled down (biotin) or not (lysate) with streptavidin–agarose beads, separated by SDS-PAGE and revealed by Western blotting with an anti-CD4 antibody. The molecular mass marker is indicated on the left. (d) Immunofluorescence analysis of the effect of the position of the ALALAL insertion on the subcellular localization of CD4–TM1prM.
CD4 and CD4–TM1prM(AL)3, CD4–TM1prM–G was barely detected at the plasma membrane (Fig. 4c). Together, these data indicate that the ER-retention signal present in TM1 of prM has a dominant effect on the VSV-G export signal.

As insertion of an ALALAL sequence in the middle of TM1 of prM alters the ER retention of this transmembrane segment, we tested whether a combination of this ALALAL insertion in TM1 of prM together with the addition of VSV-G export signal at the C terminus of TM1 of E would lead to export of prME. However, to avoid any interference with potential additional ER-retention signal(s) in the ectodomains of these proteins, this was tested in CD4–TMprM(AL)3–TM1E–G chimeric protein (Fig. 3). As shown in Fig. 3(b), CD4–TMprM(AL)3–TM1E–G was retained in the ER, despite the presence of the ALALAL insertion in TM1 of prM and an export signal at the C terminus of TM1 of E, suggesting that an additional ER-retention signal might be present in the second transmembrane passage of prM.

Identification of an ER-retention signal in TM2 of prM

Although an ER-retention signal is present in the first transmembrane segment of prM, the transmembrane region of prM is composed of two antiparallel transmembrane α-helices and we cannot exclude an additional role for the second transmembrane segment (TM2) in ER retention, as suggested above. To test this hypothesis, TM2 of prM was fused to the reporter green fluorescent protein (GFP) instead of CD4 (Fig. 5a; TM2prM–GFP). Indeed, due to the opposite orientation of this segment compared with TM1, a fusion with the ectodomain of CD4 was not possible in this case. As shown in Fig. 5(b), this chimeric protein was retained in an intracellular compartment. Furthermore, co-immunostaining with ER (calnexin) and Golgi (GM130) markers indicated that TM2prM–GFP is retained in the ER (Fig. 5b). As for ER retention mediated by TM1 of prM and E, we tested whether adding two helix turns in TM2 of prM would lead to a different subcellular localization of the TM2prM–GFP reporter construct. We therefore added an ALALAL sequence in the middle of TM2 of prM and compared the subcellular localization of this protein (TM2prM(AL)3–GFP) with that of TM2prM–GFP. Although TM2prM(AL)3–GFP was not expressed at the plasma membrane, its intracellular distribution was different from that of TM2prM–GFP (Fig. 5b). Indeed, the immunofluorescent material was concentrated predominantly in the perinuclear region. To evaluate further the subcellular localization of TM2prM(AL)3–GFP, co-immunostainings were performed with ER (calnexin) and Golgi (GM130) markers. As shown in Fig. 5(b), a strong colocalization with GM130 was observed, indicating that a large proportion of TM2prM(AL)3–GFP is localized in the Golgi. Together, these data indicate that TM2 of prM also contains an ER-retention function.

![Fig. 5. Identification of an ER-retention signal in TM2 of prM.](image-url)

(a) Schematic diagrams of wild-type or mutated TM2 of prM in fusion with GFP. The sequences of wild-type and mutated TM2 of prM are indicated below the diagrams and the putative minimal transmembrane sequence of the wild type (Cocquerel et al., 2000) is highlighted in grey. Mutated TM2 (TM2prM(AL)3) had the sequence ALALAL added as shown. It should be noted that, as TM2 of prM works as a signal sequence, the C-terminal residue of TM2 of prM was replaced by an Arg residue to avoid cleavage between TM2 of prM and GFP. (b) Confocal immunofluorescence analysis of the localization of TM2prM–GFP and TM2prM(AL)3–GFP. HeLa cells transfected with the appropriate plasmids were processed for immunofluorescence staining for the detection of calnexin (CNX) and GM130 as ER and Golgi markers, respectively (middle panels). TM2prM–GFP and TM2prM(AL)3–GFP were detected by GFP fluorescence. Colocalization was visualized by overlay. Representative images of cells expressing TM2prM–GFP and TM2prM(AL)3–GFP are shown.
Localization signals in TM1 of E and prM induce a static retention in the ER

Although CD4–TM1E and CD4–TM1prM were localized in the ER at steady state, our data do not exclude transport to the cis Golgi followed by recycling to the ER, as observed for luminal proteins containing a C-terminal sequence of the prototype KDEL (Lewis & Pelham, 1992; Martire et al., 1996; Townsley et al., 1993). To answer this question, cells expressing CD4–TM1E or CD4–TM1prM were treated with nocodazole. This agent disrupts microtubules, leading to disintegration of the Golgi and interruption of traffic between the Golgi, the ER–Golgi intermediate compartment (ERGIC) and the ER (Cole et al., 1996; Saraste & Svensson, 1991). The fate of CD4–TM1E and CD4–TM1prM was compared with that of protein disulfide isomerase (PDI), an ER-resident protein that contains a KDEL signal for retrieval. CD4–TM1E, CD4–TM1prM and PDI showed an ER pattern by double-label immunofluorescence on cells without treatment (Fig. 6a, b). After treatment for 3 h with 20 mM nocodazole, much of the PDI was concentrated in scattered bright spots (Fig. 6). The same result was obtained after treatment for longer times, up to 7 h (data not shown). However, such concentrated spots were not observed for CD4–TM1E or CD4–TM1prM (Fig. 6a, b). An ERGIC marker, ERGIC-53 (Schweizer et al., 1988), showed the expected perinuclear staining without treatment, which changed to more punctate staining after treatment with nocodazole (Fig. 6c). The punctate staining of ERGIC-53 after treatment showed only a minor colocalization with that of PDI (Fig. 6d). Under these conditions, PDI may be concentrating at ER exit sites, as suggested by Shenkman et al. (1997). Together, these data suggest that CD4–TM1E and CD4–TM1prM do not leave the ER and are probably retained in this compartment by static retention.

DISCUSSION

Here, we characterized ER-retention signals present in the transmembrane segments of YFV envelope proteins, prM.
and E. The prME heterodimer contains at least three ER-retention signals. Indeed, we identified two ER-retention signals in the transmembrane region of prM and at least one in the transmembrane region of E. In the absence of any known motif, localization of flavivirus envelope proteins probably results from the properties of their transmembrane domains and their interaction with the membranes. It has also been postulated that the length of the hydrophobic transmembrane domains are a distinguishing characteristic of proteins localized to the membrane of the Golgi apparatus or plasma membrane: shorter transmembrane domains are characteristic for the Golgi and longer ones for the plasma membrane (Bretscher & Munro, 1993). In the context of proteins retained in the ER, increasing the length of transmembrane domains has also been shown to disrupt ER retention of some proteins (Pedrazzini et al., 1996; Szczesna-Skorupa & Kemper, 2000; Yang et al., 1997), indicating that similar transmembrane-based sorting exists in the ER. Similarly, increasing the length of TM1 of E leads to export to the plasma membrane. In the case of TM2 of prM, adding two turns to the transmembrane segment also led to export of the reporter protein out of the ER; however, the protein did not go to the plasma membrane, but was mainly retained in the Golgi apparatus. This difference between TM1 and TM2 is probably a result of differences in the length of the transmembrane segments. In the case of TM1 of prM, adding two turns to the transmembrane segment led to export of the reporter protein only when the amino acids were inserted in the middle of the transmembrane segment, suggesting that, in addition to the length, specific residues present in this domain can also play a role in ER retention.

The addition of a diacidic export motif can outweigh the effect of the ER-retention signal of TM1 of E. As for some other proteins (Pedrazzini et al., 1996; Szczesna-Skorupa & Kemper, 2000; Yang et al., 1997), signals for ER retention are present in the transmembrane domains of YFV envelope proteins. Importantly, export out of the ER can also be modulated by the presence or absence of specific export motifs, such as the well-characterized diacidic motif (Nishimura & Balch, 1997). Interestingly, adding such a diacidic export motif at the C terminus of TM1 of E led to export to the cell surface, indicating that the diacidic motif is dominant over the ER-retention signal present in TM1 of E. ER localization of a protein can potentially be due either to retrieval by retrograde transport carriers or to exclusion from anterograde transport carriers. However, it has recently been shown that proteins with a short transmembrane domain are excluded from ER exit sites, indicating that physicochemical features of the transmembrane domain influence sorting of membrane proteins by simple receptor-independent mechanisms based on partitioning (Ronchi et al., 2008). A transmembrane protein could thus seek ER subcompartments in which the lipid composition better matches its length/hydrophobicity. Although detailed information is lacking so far, lipid compositional differences between ER exit sites, transport carriers and the bulk of the ER are very likely to exist. In this context, our data with TM1 of E suggest that the presence of an export motif might force the reporter protein to be redirected to the ER exit sites to be actively incorporated into transport carriers. ER localization of CD4–TM1E can therefore be seen as passive retention in an ER subcompartment due to suboptimal physicochemical properties of the transmembrane domain. Interestingly, increasing the length can modify the properties of the TM domains, which probably allows the protein to diffuse into different subcompartments of the ER and be passively incorporated into transport carriers.

The addition of a diacidic export motif cannot outweigh the effect of the ER-retention signal of TM1 of prM. This indicates that the ER-retention signal present in TM1 of prM is dominant over the diacidic motif. These data also suggest that, in this context, the presence of an export motif cannot force the reporter protein to be redirected to the ER exit sites to be actively incorporated into transport carriers. Differences in the amino acid composition between TM1 of prM and TM1 of E may potentially explain their difference in competing with the diacidic export motif. Furthermore, we cannot exclude the possibility that TM1 of prM is retained in the ER by a receptor interacting with this transmembrane region. If it is the case, the insertion of the ALALALAL sequence would alter the motif involved in the interaction with the ER receptor.

YFV envelope proteins are multi-spanning membrane proteins. Indeed, the C termini of prM and E form two antiparallel transmembrane α-helices (Zhang et al., 2003a, b). This unusual topology for viral envelope proteins is due to the fact that YFV proteins are synthesized as a polyprotein, which is cleaved co-translationally by a cellular signal peptidase between prM and E and between E and the downstream protein NS1 (reviewed by Lindenbach et al., 2007). In this context, the second transmembrane segment of prM and E acts as a signal for reinitiation of translocation (signal-like sequence) of the downstream proteins. By being retained as transmembrane sequences, the signals of reinitiation of translocation present at the C termini (TM2) of prM and E can also play additional functions. Here, we show that TM2 of prM also contributes to the ER retention of prM.

Interestingly, the envelope glycoproteins of hepatitis C virus (HCV), another member of the family Flaviviridae, also contain ER-retention motifs in their transmembrane domains (Cocquerel et al., 2002). However, for HCV, the retention mechanism involves charged residues of the transmembrane domains, in contrast to YFV envelope protein, for which the mechanism of ER retention appears to rely primarily on the length of the transmembrane segments and not on the presence of charged residues. It is striking that both viruses use the transmembrane domains as retention motifs for their envelope proteins, but that they use different cellular mechanisms for this function. This highlights the peculiar importance of the transmem-
brane domains of the envelope proteins for the biology of the viruses of this family.

As a result of good conservation in the organization of the transmembrane domains in prM and E of members of the genus Flavivirus (Cocquerel et al., 2000), ER-retention signals, similar to those characterized in this work, are likely to be present in the other flaviviruses. However, differences in the strength of such signals can exist, as highlighted recently (Hsieh et al., 2008). The flavivirus envelope protein heterodimer prME plays a major role in the budding process leading to the formation of the virus particle (reviewed by Heinz & Allison, 2000). After budding in the ER, the virus particles are transported through the normal secretory pathway (Lorenz et al., 2003; Mackenzie & Westaway, 2001). The presence of several ER-retention signals is important for the ER localization of prME, a major player of the budding process. Furthermore, the presence of a dominant ER-localization signal in prM might also direct the prME heterodimer to a subcompartment of the ER in which budding takes place. In conclusion, the transmembrane domains of the flavivirus envelope proteins are multifunctional. Their structure and topology are important for the processing of the viral polyprotein and for the budding process (Op De Beeck et al., 2003). In addition, we show here that they contain ER-localization signals that are likely to be essential for the budding process to take place in the appropriate compartment.

METHODS

Cell culture. HeLa cells obtained from the ATCC (Manassas, VA, USA) were grown in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (PAA Laboratories).

Antibodies. mAbs 2D12 (anti-E; ATCC CRL-1689) and OKT4 (anti-CD4; ATCC CRL-8002) were from ATCC. mAb P5D4, directed against VSV-G, was from Sigma, the polyclonal anti-CD4 was from Santa Cruz and the anti-PDI, anti-calnexin and anti-calreticulin antibodies were from Stressgen. Mouse anti-GM130 mAb was purchased from BD Biosciences. Alexa 488- and Alexa 546-conjugated goat anti-rabbit and anti-mouse IgG were from Molecular Probes. Cy3-conjugated goat anti-rat IgG was from Jackson ImmunoResearch.

Plasmids. Plasmids expressing chimeric proteins were constructed by using standard methodology (Sambrook et al., 1989) in the context of a pcDNA3.1 + vector (Invitrogen). YFV sequences were amplified from plasmid pAP5, which contains the sequence encoding the structural proteins of the YFV 17D-204-Pasteur strain (Despres et al., 1987). Mutants were constructed by sequential PCR steps as described by Ausubel et al. (2000) using the high-fidelity Deep Vent DNA polymerase (New England Biolabs). The mutants were then assembled by a second PCR amplification and verified by sequencing (Ausubel et al., 2000). For some constructs, a VSV-G cytosolic tail (VGLHCICKLHKTQKYTYDIEMNRGLGK) containing the DIE export motif was fused at the C terminus of chimeric proteins. In some of these constructs, the export signal of the VSV-G cytosolic tail was mutated by replacing all the amino acids of the YTDIEM sequence by Ala residues (Sevier et al., 2000). The sequence encoding the TM2 of prM was generated by PCR and cloned into the pEGFP-N1 plasmid (Clontech) to express TM2 of prM in fusion with GFP. As TM2 functions as a signal peptide, we abolished the cleavage between TM2 and GFP by replacing the C-terminal amino acid of TM2 by an Arg residue, which is known to abolish signal peptide processing. The CD4–TMprM–TM1E–G construct was made to produce a fusion protein devoid of prME ectodomains and containing the ectodomain of CD4 and a VSV-G cytosolic tail. For this construct, the C-terminal amino acid of TM2 of CD4 was replaced by an Arg residue to avoid cleavage between the TM2 of prM and the TM1 of E. Furthermore, an influenza haemagglutinin (HA) epitope (YPYDVPDYA) surrounded by two glycine residues was inserted between the transmembrane domain of prM and the first transmembrane domain of E. All of the CD4 constructs contained the CD4 signal peptide and prME contained the prM signal sequence.

Indirect immunofluorescence microscopy. HeLa cells were grown on 12 mm glass coverslips and transfected with the appropriate plasmid using FuGENE 6 (Roche). At the indicated time, cells were fixed with 3 % paraformaldehyde and then permeabilized with 0.1 % Triton X-100 (Sigma-Aldrich) in PBS. Immunolabelling and confocal microscopy were performed as described previously (Rouillé et al., 2006).

Detection of cell-surface biotinylated proteins. Cell-surface biotinylation was performed as described previously (Belouzard et al., 2004).

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