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An unstable transmembrane segment in the cystic fibrosis transmembrane conductance regulator

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The cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel with 12 membrane-spanning sequences, undergoes inefficient maturation in the endoplasmic reticulum (ER). Potentially charged residues in transmembrane segments may contribute to this defect in biogenesis. We demonstrate that transmembrane segment 6 of CFTR, which contains three basic amino acids, is extremely unstable in the lipid bilayer upon membrane insertion *in vitro* and *in vivo*. However, two distinct mechanisms counteract this anchoring deficiency: (i) the ribosome and the ER translocon co-operate to prevent transmembrane segment 6 from passing through the membrane co-translationally; and (ii) cytosolic domains of the ion channel post-translationally maintain this segment of CFTR in a membrane-spanning topology. Although these mechanisms are essential for successful completion of CFTR biogenesis, inefficiencies in their function retard the maturation of the protein. It seems possible that some of the disease-causing mutations in CFTR may reduce the efficiency of proper membrane anchoring of the protein.

Keywords: cystic fibrosis/endoplasmic reticulum/membrane protein/translocation

Introduction

Cystic fibrosis (CF) is caused by a defect in the cystic fibrosis transmembrane conductance regulator (CFTR), a cell surface chloride channel (Welsh and Smith, 1993). CFTR is a member of the ABC family of transmembrane transporters, and is largely regulated via its three cytosolic domains (Riordan *et al.*, 1989; Anderson and Welsh, 1992). Two of these domains affect channel activity by binding and hydrolysing ATP, and the third, known as the regulatory (R) domain, alters ion transport in a phosphorylation-dependent manner (Rich *et al.*, 1991; Baukrowitz *et al.*, 1994; Gunderson and Kopito, 1995). The two membrane domains of CFTR consist of six transmembrane helices each and presumably form the chloride channel (Figure 1; Chang *et al.*, 1994).

The biogenesis of CFTR is inefficient (Cheng *et al.*, 1990): during insertion into the membrane of the endoplasmic reticulum (ER), CFTR interacts with the cytosolic chaperones Hsc70/Hsp40 and Hsp90, as well as with the ER chaperone calnexin (Yang *et al.*, 1993; Pind *et al.*, 1994; Loo *et al.*, 1998; Meacham *et al.*, 1999). After an undefined maturation event, the chaperones release CFTR, and the protein moves through the secretory pathway, ultimately reaching the plasma membrane. However, an estimated 50–70% of wild-type CFTR fails to mature and does not transit the secretory pathway (Lukacs *et al.*, 1994; Ward and Kopito, 1994). Several mutations associated with CF further reduce the ability of CFTR to exit the ER; for example, deletion of Phe508 (ΔF508) results in virtually complete retention of the mutant protein in the secretory compartment (Cheng *et al.*, 1990; Sheppard *et al.*, 1995). Proteolytic removal of immature CFTR in the ER occurs predominantly through the ubiquitin–proteasome system (Jensen *et al.*, 1995; Ward *et al.*, 1995). In the present study we examine an aspect of the biogenesis of CFTR that may contribute to the inefficiency of its maturation.

The primary sequence of CFTR indicates that eight of its 12 predicted transmembrane segments contain potentially charged amino acids (Figure 1A). Insertion of an acidic or basic amino acid within a transmembrane segment can cause intracellular retention and a marked reduction in the stability of the entire protein (Davis and Hunter, 1987; Bonifacino *et al.*, 1990, 1991). Such residues have the strongest influence on trafficking and stability of a protein when they are part of a membrane anchor sequence containing fewer than 22 amino acids and reside centrally within the lipid bilayer. These destabilizing effects are less pronounced when the length of the transmembrane segment increases or the charged residue is situated closer to the lipid head groups (Bonifacino *et al.*, 1991; Lankford *et al.*, 1993). Charged residues can impair the anchoring function of a transmembrane segment, thus allowing protein domains normally exposed to the cytosol to be translocated into the ER lumen (Shin *et al.*, 1993). However, pairing of basic residues in one transmembrane segment with acidic residues in a second transmembrane segment can mask the retention and degradation determinants, thereby allowing completion of maturation, including transit through the secretory pathway (Cosson *et al.*, 1991; Tiwari-Woodruff *et al.*, 1997).

Transmembrane segment 6 (Tm6) of CFTR could influence the maturation of the ion channel if its three basic residues, arginine 334, lysine 335 and arginine 347 (Figure 1B), were not stabilized in the lipid bilayer. Arginine 347 has been shown to form a salt bridge with an aspartate located in another transmembrane segment, suggesting that pairing of acidic and basic residues from different transmembrane segments of CFTR could promote maturation of the protein similarly to what has been observed with the T cell receptor (Cotten and Welsh, 1999). However, several lines of evidence indicate that this does not occur. Mutating any one of the three basic amino acids in Tm6 of CFTR to neutral or acidic residues alters the pore properties of the

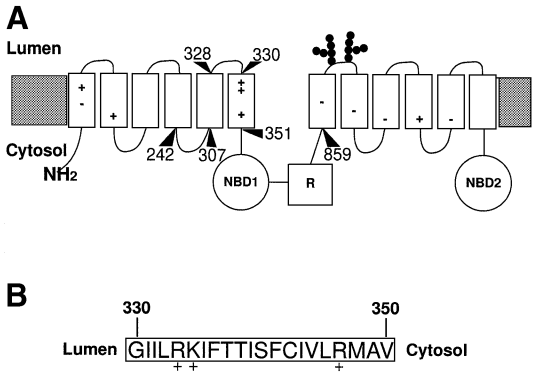

Fig. 1. Schematic representation of CFTR. (**A**) CFTR has 12 transmembrane segments and three large cytosolic domains. Two domains bind ATP (NBD1 and NBD2) and the regulatory (R) domain modulates channel activity depending on its phosphorylation status. Numbered arrowheads indicate various positions in the primary sequence of CFTR. The approximate locations of basic and acidic residues in the transmembrane segments are marked by + and – symbols, respectively. Filled circles represent two N-linked glycans on the lumenal loop connecting Tm7 and Tm8. (**B**) The amino acids predicted to form Tm6 are shown.

channel without grossly affecting its biogenesis (Anderson *et al.*, 1991; Sheppard *et al.*, 1993). Moreover, Tm6 can be stabilized in the absence of four of the five acidic intramembrane residues of CFTR which are located in transmembrane segments 1, 7, 8, 9 and 11 (Figure 1A). A fragment of CFTR, containing transmembrane segments 1–6 plus the cytosolic NBD1 and R domains, forms active ion channels at the surface of mammalian cells. Expression of this construct in *Xenopus* oocytes or in mammalian cells indicates that it is at least as stable as the full-length nascent protein (Sheppard *et al.*, 1994; Xiong *et al.*, 1997; Meacham *et al.*, 1999). Thus, the positively charged residues in Tm6 are important for the function of CFTR as a chloride channel, yet they do not need to form ion pairs in order to stabilize Tm6 in the membrane and to permit the complete maturation of the protein.

Here we demonstrate that despite the ability of Tm6 to stop translocation in the ER membrane, this segment fails to behave as a proper membrane anchor. Instead, the ribosome–ER translocation machinery and the cytosolic domains of CFTR co-operate to inhibit slipping of this transmembrane segment into the ER lumen. The incomplete effectiveness of these processes may contribute to the general inefficiency of CFTR maturation.

Results

Tm6 fails to act as an efficient anchor sequence in the ER

The aim of this study was to gain insight into the mechanisms that contribute to the inefficient biogenesis of CFTR in the ER. Since the presence of potentially charged residues in membrane anchors of integral membrane proteins can inhibit their cell surface expression, we considered the possibility that Tm6, which contains three positively charged residues and precedes the NBD1 domain (Figure 1), may contribute to the inefficient maturation of authentic CFTR.

A detailed analysis of the membrane anchor properties of Tm6 of CFTR was performed *in vivo* in the yeast

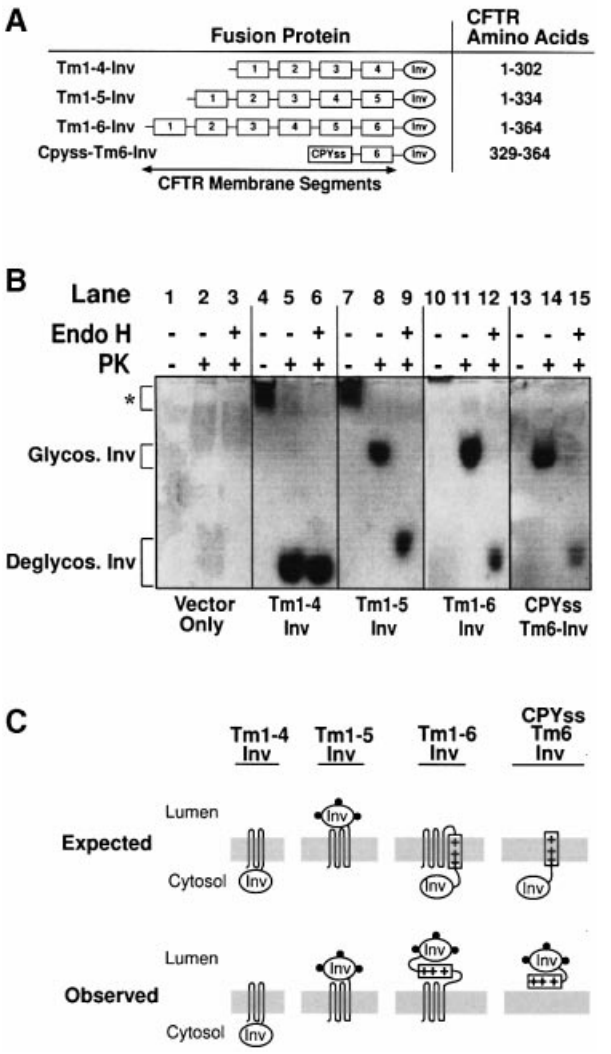

Fig. 2. Evaluation of the ability of Tm6 of CFTR to anchor in the lipid bilayer. (**A**) Schematic representation of fusion proteins, containing various parts of CFTR linked to the N-terminus of full-length invertase for expression in *S.cerevisae*. Numbered boxes indicate transmembrane segments of CFTR, invertase is indicated by Inv and CPYss represents the carboxypeptidase Y signal sequence (see also Figure 6). (**B**) A reporter protein immediately following Tm6 of CFTR is translocated into the lumen of the yeast ER *in vivo*. Membranes isolated from yeast expressing the proteins described in (A) were solubilized in 1% Triton X-100 and the lysates analysed on a native gel. Their location in the gel, marked by an asterisk, was visualized by staining for invertase activity (lanes 1, 4, 7, 10 and 13). Following detergent lysis, samples were incubated with (lanes 3, 6, 9, 12 and 15) or without (lanes 2, 5, 8, 11 and 14) Endo H and then treated with proteinase K. The gel mobility of proteolytically released invertase was markedly affected by the presence (Glycos. Inv) or absence (Deglycos. Inv) of N-linked glycans. (**C**) Schematic representation of the expected and observed membrane topologies for the CFTR–invertase fusion proteins expressed in yeast cells. Tm segments 1–5 are represented as lines, and the basic residues in Tm6 are depicted as + symbols. N-linked glycans are marked by solid black circles.

Saccharomyces cerevisiae. Various fusion constructs in which full-length invertase was linked to the C-termini of different transmembrane segments of CFTR (Figure 2A) were expressed in a yeast strain lacking endogenous invertase. Invertase is produced with or without a signal sequence and hence is expressed in the cytosol or in the secretory pathway. When this enzyme enters the ER

lumen, it acquires ~10 N-linked glycans (Reddy *et al.*, 1988). These modifications cause the protein to exhibit a much slower rate of migration on a native polyacrylamide gel compared with the non-glycosylated, cytosolic form of invertase (Carlson *et al.*, 1981; Kaiser and Botstein, 1986). Consequently, the orientation of a transmembrane segment across the lipid bilayer can be determined by examining the glycosylation status of invertase fused to the C-terminus of the membrane anchor (Wilkinson *et al.*, 1996). The predicted and observed topologies of the fusion proteins analysed are shown in Figure 2C.

Membranes were prepared from yeast constitutively expressing the different fusion proteins, washed with high salt and EDTA, and solubilized in non-ionic detergent. The invertase contained in these extracts did not migrate significantly into the gel, unless it was proteolytically separated from the respective CFTR segments by treatment with proteinase K, utilizing the fact that folded invertase is intrinsically protease resistant (Figure 2B, lanes 4 and 5, 7 and 8, 10 and 11, 13 and 14). As expected, the invertase reporter was not glycosylated when attached to the C-terminus of Tm4, indicating its localization in the cytosol. This topology was determined on the basis of the fast migration of the protease-released invertase moiety on native PAGE and the absence of a shift in migration upon treatment with endoglycosidase H (Endo H), which removes N-linked glycans from proteins (Figure 2B, lanes 4–6). Invertase was glycosylated, however, when attached to the C-terminus of Tm5 (Figure 2B, lanes 7–9), as indicated by the slow migration on the gel of the protease-released invertase and its accelerated mobility following Endo H treatment. Strikingly, in contrast to the predicted topology, invertase was also glycosylated when attached to the C-terminus of Tm6 (Figure 2B, lanes 10–12). The same observation was made for the construct CPYss–Tm6–Inv (Figure 2A), in which the signal sequence of carboxypeptidase Y precedes Tm6 and invertase (Figure 2B, lanes 13–15, and Figure 6). These results support the hypothesis that Tm6 of CFTR fails to act as a proper anchoring segment of the protein in the ER membrane. Instead, this segment slips through the lipid bilayer and allows the translocation of subsequent protein domains into the ER lumen.

In order to verify that the results obtained in yeast accurately reflected the events in a mammalian system, CFTR constructs were translated in a rabbit reticulocyte lysate supplemented with canine pancreatic microsomes. Translation of constructs with multiple transmembrane segments was relatively inefficient. We therefore analysed fusion constructs containing only Tm5 and Tm6 of CFTR, followed by the N-terminal 256 amino acids of mature invertase (with C-terminal myc and His_6 tags) (Figure 3A). This fragment of invertase does not fold into a protease-stable structure and therefore does not exhibit an intrinsic resistance to proteinase K (Figure 3C), in contrast to the full-length invertase molecule that is used in the fusion proteins for expression in yeast (see Figures 2 and 5). Proper insertion of Tm5 with its N-terminus facing the cytosol appears to follow the positive inside rule (von Heijne and Gavel, 1988; Hartmann *et al.*, 1989; von Heijne, 1989). Removing the polypeptide sequence immediately preceding Tm5, which contains four positively charged but no negatively charged amino acids (Figure 3B), caused

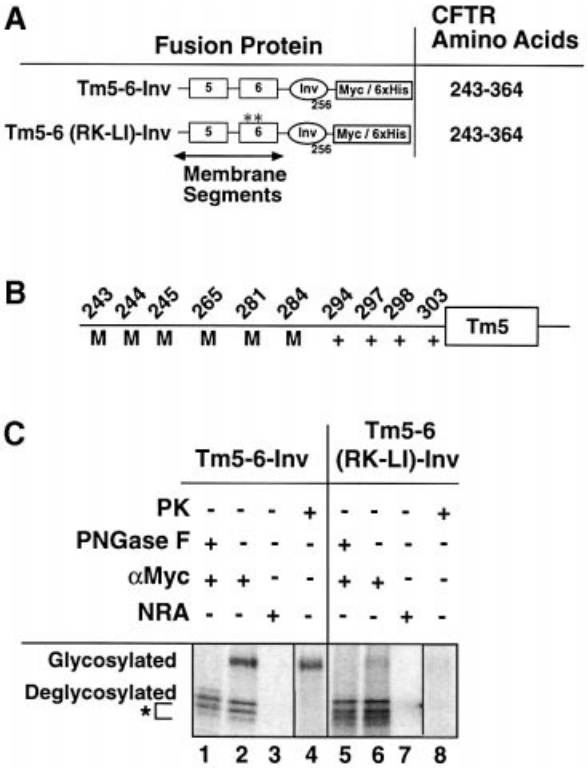

Fig. 3. Inefficient membrane anchoring of Tm6 of CFTR upon expression in a mammalian *in vitro* translation/translocation system. (**A**) Schematic representation of CFTR-derived constructs translated in a reticulocyte lysate supplemented with canine pancreatic microsomes. Inv_{256} is a fragment of invertase containing the N-terminal 256 residues of the mature protein (513 residues). (**B**) The sequence preceding Tm5 starting from residue 243 of CFTR. Various positions of methionines and positively charged residues are indicated. (**C**) Translation and translocation into microsomes of constructs containing wild-type or doubly mutated, R334L and K335I, Tm6 were analysed by immunoprecipitation with anti-myc antibody (lanes 1, 2, 5 and 6) or a non-relevant control antibody (NRA) (lanes 3 and 7) from TX-100 extracts of the isolated microsomes. The anti-myc immunoprecipitates were further incubated with or without PNGaseF (lanes 1 and 5). Proteinase K (100 μg/ml, 30 min on ice) was added to the reactions prior to isolation of microsomes (lanes 4 and 8).

a significant proportion of a control protein to insert with an inverted topology (data not shown). Therefore the entire segment of CFTR (residues 243–307) between Tm4 and Tm5 was included as part of the fusion proteins. This segment also contains six methionines (Figure 3B), which gave rise to internal initiation of translation (see asterisk in Figure 3C).

Immunoprecipitation with an antibody directed against the C-terminal myc tag confirmed that the translation products obtained were indeed derived from construct Tm5-6–Inv (Figure 3C, lane 2). No material copurified with a non-relevant antibody (Figure 3C, lane 3). The most slowly migrating band exhibited a molecular weight equal to that predicted for the full-length protein when glycosylated on the invertase fragment. This product was protected against digestion with proteinase K (Figure 3C, lane 4) and exhibited an increased gel mobility following treatment with the enzyme peptide:*N*-glycosidase F (PNGase F) (Figure 3C, lanes 1 and 2), which removes N-linked glycans. These results demonstrated that the invertase moiety of this translation product resided in the microsomal lumen. In contrast, the invertase moiety of

the more rapidly migrating protein species remained exposed to the cytosol, as demonstrated by its sensitivity to digestion by proteinase K (Figure 3C, lane 4) and its unaltered gel mobility following treatment with PNGase F (Figure 3C, compare lanes 1 and 2). All of the glycosylated and a significant fraction of the non-glycosylated protein was associated with the microsomal membrane in a salt-resistant manner, as demonstrated by separating microsomes and cytosol by gel filtration of translation extracts (Young *et al.*, 1995; data not shown). Thus, construct Tm5-6–Inv acquired dual topologies in the ER membrane, indicating that Tm6 failed to behave as an effective membrane anchor in the mammalian ER, consistent with the results obtained upon expression of CFTR constructs in yeast.

The contribution of basic amino acids to the poor stop-translocation activity of Tm6 was evaluated by mutating two of these residues to hydrophobic amino acids. Arginine 334 was changed to leucine and lysine 335 to isoleucine, generating construct Tm5-6(RK-LI)–Inv (Figure 3A). Only a very small amount of the invertase in this construct translocated into the ER lumen and was glycosylated (Figure 3C, lanes 5 and 6). The vast majority of the invertase was exposed on the cytosolic face of the microsomal membrane, based on its accessibility to proteinase K (Figure 3C, lane 8). Thus, as expected, changing residues arginine 334 and lysine 335 in Tm6 to hydrophobic amino acids causes a marked stabilization of Tm6 in the lipid bilayer, preventing it from entering the ER lumen together with subsequent portions of the polypeptide.

Cytosolic domains of CFTR oppose post-translational translocation of Tm6

Tm6 of CFTR is followed by the cytosolic NBD1 and R domains. To analyse the ability of these domains to support the correct membrane anchoring of Tm6, construct Tm1–6-NBD1-R–Inv (Figure 4A) was expressed in yeast. Interestingly, glycosylated and non-glycosylated invertase was detected in the salt-washed membranes of these cells in approximately equimolar amounts (Figure 4B), indicating that the fusion protein assumed a mixture of membrane topologies in which invertase (and presumably the preceding NBD1 and R domains of CFTR) was either translocated into the ER lumen or acquired its correct cytosolic location, as shown schematically in Figure 4C. In contrast, expression of the construct Tm1–6–Inv resulted exclusively in the formation of glycosylated invertase (Figure 2B). Thus, the presence of the subsequent cytosolic domains, NBD1 and R, partially corrects the defect of Tm6 in faithfully anchoring the protein in the membrane.

The ability of the cytosolic domains of CFTR to prevent or retard the translocation of Tm6 suggested that the failure of this segment to anchor in the membrane occurred either during or after synthesis of the NBD1 and R domains. To explore this possibility, construct Tm1–6–Ura3 was generated, which contains Tm1 through Tm6 of CFTR fused to the N-terminus of the enzyme Ura3 (Figure 5A). When yeast is grown in media lacking uracil, this enzyme becomes essential as it is part of the biosynthetic pathway that generates uracil in the cytosol. Ura3 can be targeted to the ER lumen via co- or post-translational pathways, effectively making the cell devoid of its activity (Johnsson and Varshavsky, 1994; Ng *et al.*, 1996). To sustain growth,

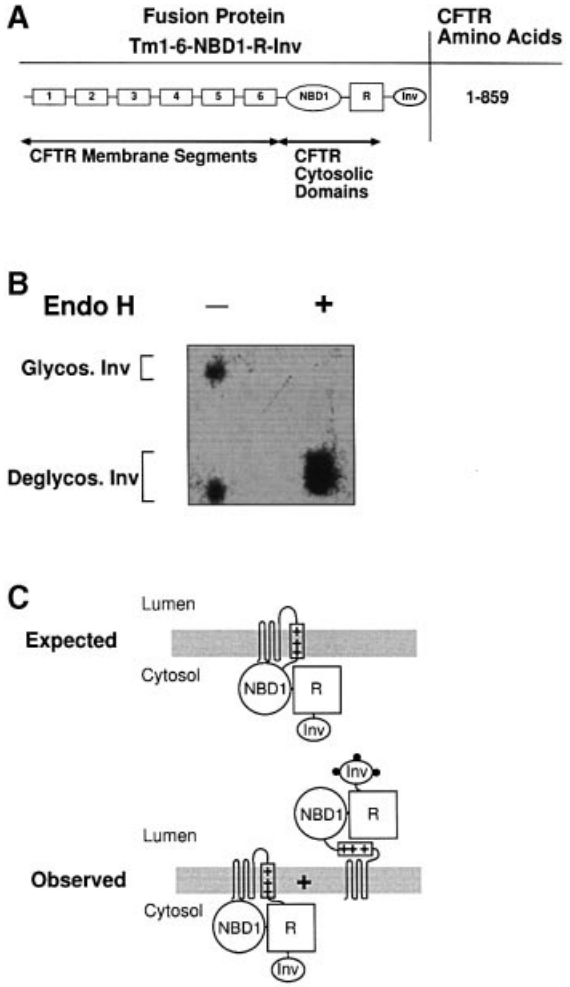

Fig. 4. CFTR sequences following Tm6 markedly inhibit translocation into the ER lumen of yeast. (**A**) Schematic representation of the Tm1–6-NBD1-R–Inv fusion protein expressed in yeast. (**B**) Membranes isolated from yeast expressing the fusion protein were lysed in detergent, treated with proteinase K and analysed for the presence of glycosylated and non-glycosylated invertase by native gel assay exactly as in Figure 2B. (**C**) Schematic representation of the expected and observed membrane topologies for the Tm1–6-NBD1-R–Inv fusion proteins expressed in yeast cells. Symbols are the same as in Figure 2C.

the Ura3 protein must have a half-life of ~10 min in the cytosol (Dohmen *et al.*, 1994). Control experiments confirmed that cells lacking an endogenous Ura3 gene and expressing the fusion protein Tm1–6–Inv did not grow in the absence of uracil. However, growth on uracil-free medium was observed when these cells produced the Tm1–6–Ura3 protein (Figure 5B). Thus, upon expression of this construct, at least some of the Ura3 protein must be transiently exposed to the cytosol. Given that upon expression of Tm1–6–Inv all invertase is translocated into the ER lumen (Figure 2B), translocation of Tm6 and of the cytosolic domain that follows it is likely to occur post-translationally, at least for a portion of the molecules.

Because only small amounts of Ura3 are required to sustain the growth of yeast, a quantitative cellular assay was developed to evaluate the timing of translocation of Tm6 relative to its translation. Construct Tm1–6–TEV–Inv was generated, which contains a tobacco etch virus (TEV) protease cleavage site between Tm1–6-CFTR and

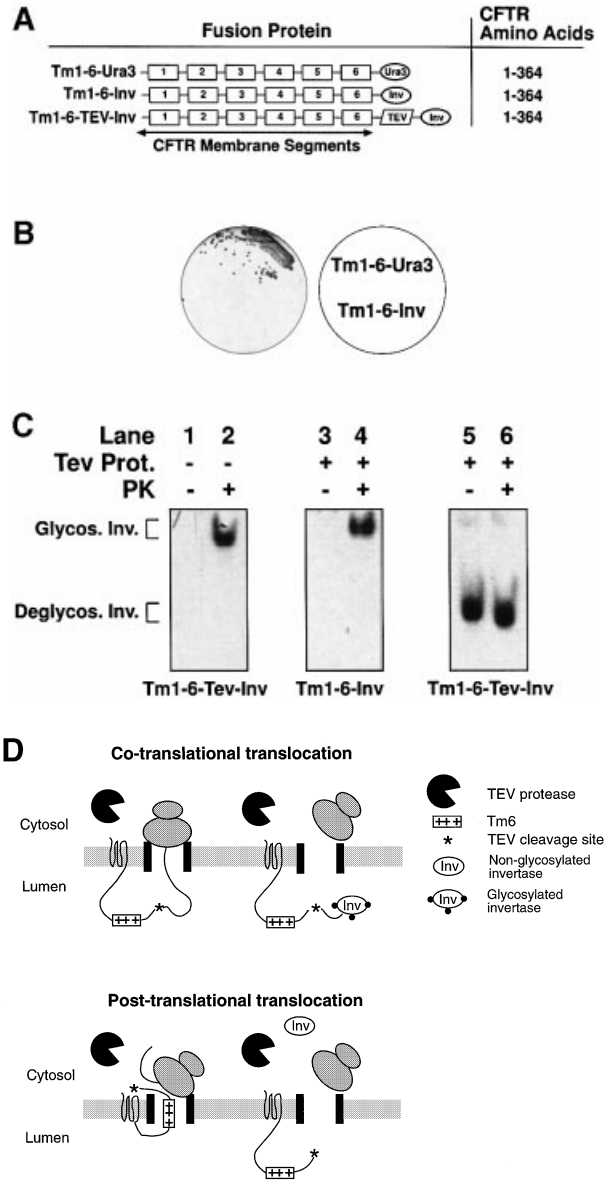

Fig. 5. Polypeptide sequences following Tm6 are exposed to the cytosol prior to their translocation into the ER lumen. (**A**) Schematic representation of fusion proteins containing Tm1–Tm6 of CFTR linked to the N-terminus of either Ura3 or invertase. In a third construct a TEV protease cleavage site was introduced between invertase and CFTR. (**B**) Yeast expressing constructs Tm1–6–Inv or Tm1–6–Ura3 were streaked on plates lacking uracil and incubated for 5 days at 30°C. (**C**) Whole cell lysates of yeast expressing the Tm1–6–Inv (lanes 3 and 4) and Tm1–6–TEV–Inv (lanes 1, 2, 5 and 6) were examined for the presence of glycosylated and non-glycosylated invertase as described in Figure 2B. TEV protease was either co-expressed (lanes 3–6) or not (lanes 1 and 2). Proteinase K treatment of cell lysates (lanes 2, 4 and 6) was necessary to visualize invertase that was not cleaved from the fusion protein by the TEV protease. (**D**) Schematic description of the TEV protease assay described in Figure 6C. Tm segements 1–5 are represented as lines, and the basic residues in Tm6 are marked as + symbols.

invertase (Figure 5A). If Tm6 of CFTR is retained in the lipid bilayer during translation and reaches the ER lumen only post-translationally, the cleavage site should become accessible to TEV protease that is expressed in the cytosol (see Figure 5D). Cleavage at the TEV site would then cause the production of cytosolic, non-glycosylated invertase. However, should Tm6 fail to stop translocation and transit the lipid bilayer co-translationally, the TEV cleavage site should be protected from cytosolic TEV protease by the tight seal that exists between the ER translocon and the ribosome during co-translational translocation (Crowley *et al.*, 1993; Johnsson and Varshavsky, 1994) (Figure 5D). Consequently, invertase would be exposed to the lumen of the ER and would receive N-linked glycans.

In order to exclude the possibility that insertion of the TEV protease cleavage site itself did not influence the topology of the fusion protein, construct Tm1–6–TEV–Inv was expressed in cells not expressing the protease. Again, the invertase reporter efficiently entered the ER, based on the observation of exclusively glycosylated protein that migrated in the native gel after its removal from the rest of the protein by proteinase K (Figure 5C, lanes 1 and 2; also see Figure 2B). When construct Tm1–6–Inv, lacking the TEV cleavage site, was co-expressed with the TEV protease in yeast, glycosylation of invertase also occurred with undiminished efficiency (Figure 5C, lanes 3 and 4). Thus, the presence of neither the TEV protease nor the protease cleavage site has an effect on the topology of the fusion protein. Strikingly, in cells producing both the TEV protease and construct Tm1–6–TEV–Inv, essentially only non-glycosylated invertase was produced, indicating that the protein was prevented from reaching the ER lumen (Figure 5C, lanes 5 and 6). These cellular assays demonstrate that Tm6 of CFTR does not pass through the ER membrane co-translationally, as would be expected for a non-membrane anchor segment. Rather, Tm6 stops translocation transiently, allowing subsequent portions of the polypeptide to be exposed to the cytosol (Figure 5D). Slippage of this segment through the membrane occurs only during or after the synthesis of cytosolic domains that follow it. This mechanism also explains how the presence of these domains can increase the correct membrane anchoring of Tm6.

Presence of Tm6 can cause protein retention in the secretory pathway

Despite the anchoring defect observed in Tm6, CFTR at the plasma membrane has been shown to assume only the correct topology depicted in Figure 1A (Chang *et al.*, 1994). This suggests that an intracellular retention mechanism acts on the fraction of CFTR protein that has failed to acquire the proper topology. To evaluate whether or not the presence of Tm6 itself can inhibit anterograde transport of a protein through the secretory pathway, secretion of the construct CPYss–Tm6–Inv was analysed upon expression in yeast (Figure 2A). This construct comprises the signal sequence of yeast carboxypeptidase Y (CPY) followed by Tm6 plus 14 cytosolic residues of CFTR and the complete sequence of invertase. Signal peptidase cleaves the CPY signal peptide several amino acids before the junction with the CFTR segment (Figure 6A) (Johnson *et al.*, 1987). Again, Tm6 and invertase passed efficiently into the lumen of the ER where invertase was glycosylated (see Figure 2B, lanes 13–15). However, >95% of this invertase was retained in an intracellular location and was not transported to the cell surface, in contrast to wild-type invertase which is secreted to virtually 100% (Figure 6B). In conclusion, Tm6 plus the flanking cytosolic region of CFTR can prevent secretion when translocated into the ER. Although

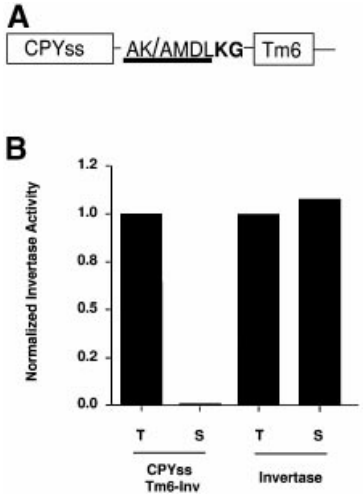

Fig. 6. Ability of Tm6 to cause retention in the secretory pathway. (**A**) Diagram of the signal sequence of carboxypeptidase Y (CPYss). The cleavage site is indicated by a backslash. Sequences corresponding to CPY are underlined and residues from CFTR are shown in bold type. (**B**) Secretion of wild-type invertase and the fusion protein CPYss–Tm6–Inv was analysed by testing for extracellular (i.e. secreted, S) and (T) total invertase activity. The total enzymic activity was set as 1.

the exact mechanism of Tm6-mediated retention remains to be explored, in the context of full-length CFTR, slippage of Tm6 into the ER lumen may prevent the secretion of CFTR that has failed to acquire the proper topology.

Discussion

We have analysed the mechanism by which transmembrane segment 6 of CFTR acquires its proper membrane-spanning orientation, thus allowing the generation of a functional protein. Because Tm6 contains several basic residues, its physical characteristics strongly favour translocation through the lipid bilayer. This inefficiency of Tm6 to function as a stop-transfer sequence would be expected to result in the complete failure of CFTR biosynthesis. However, two distinct mechanisms operate in counteracting this problem. The first acts co-translationally and is based on the residual stop-transfer property of Tm6 itself. At some point after translation of Tm6, this mechanism ceases to act and a second process, involving the NBD1 and R domains of CFTR, takes over. Presumably, these domains prevent slippage of Tm6 through the membrane during translation of the second half of CFTR (see Figure 1A) until assembly of the channel is complete.

The co-translational retention of Tm6 in the ER membrane is likely to be based on the interaction of this segment with the ER translocon (Rapoport *et al.*, 1996). This aqueous pore formed by the Sec61 protein is gated on the cytosolic side by interactions with the ribosome, whereas opening of the pore on the lumenal side is modulated by the ER chaperone BiP (Crowley *et al.*, 1994; Liao *et al.*, 1997; Hamman *et al.*, 1998). Gating is tightly regulated in order to maintain a permeability barrier between the ER lumen and the cytosol. During synthesis of an integral membrane protein, the ribosome is thought to undergo conformational changes upon recognizing a membrane anchor (Liao *et al.*, 1997). This process transmits a signal to the translocon that causes the sealing of the lumenal opening of the pore and allows the membrane anchor to interact with translocon components that mediate its integration into the lipid bilayer. Shortly afterwards, opening of the ribosome–translocon seal enables polypeptide segments following the membrane anchor to enter the cytosol (Hegde and Lingappa, 1996). Although Tm6 of CFTR is a poor membrane anchor, it nevertheless appears to be recognized by the ribosome, based on the fact that co-translational passage of Tm6 into the ER lumen is not observed. Sequences C-terminal to Tm6 are not essential to this mechanism because transient retention in the membrane is observed even when the cytosolic domains of CFTR have been replaced by invertase, which is eventually completely translocated. However, Tm6 is not efficiently integrated into the lipid bilayer upon recognition by the translocon; when the ribosome–membrane seal opens, after the insertion of Tm6 into the translocon, and translation of NBD1 and R proceeds, these domains reside first in the cytosol but can post-translationally pass through the membrane together with Tm6.

Folded protein domains, either covalently or non-covalently attached to a translocating chain, can prevent polypeptide movement across the membranes of mitochondria and the ER (Schleyer and Neupert, 1985; Eilers and Schatz, 1986; Johnsson and Varshavsky, 1994; Hegde and Lingappa, 1996). This inability of folded domains to be translocated suggests a plausible mechanism for the post-translational stabilization of Tm6 of CFTR in a membrane-spanning topology by the cytosolic NBD1 and R domains. Additionally, binding of the cytosolic chaperones Hsc70 and Hsp40 to NBD1 and R (Strickland *et al.*, 1997; Meacham *et al.*, 1999) may prevent or retard translocation of these slow folding domains even before they have been completely synthesized and reached a stable structure. On the other hand, invertase, which is also thought to fold slowly (Johnsson and Varshavsky, 1994), failed to maintain this segment of CFTR in the lipid bilayer. This observation suggests that slowing of the folding of NBD1, as may occur in the ΔF508 mutant, may provide an increased opportunity for Tm6 and subsequent cytosolic domains to enter the ER lumen, thereby perhaps reducing the efficiency of CFTR maturation.

Xenopus oocytes are known to possess a quality control mechanism in the secretory pathway which processes wild-type CFTR and the mutant ΔF508 with an efficiency very similar to that observed in mammalian cells. Expression of various CFTR truncations in *Xenopus* oocytes revealed an interesting pattern of instability (Xiong *et al.*, 1997). A construct containing the first 393 amino acids of CFTR, ending ~40 amino acids after Tm6, was unstable compared with constructs that included NBD1 or NBD1 and R. In fact, these latter constructs had a half-life very similar to that of full-length wild-type CFTR. Similar results were obtained in a mammalian cell culture system. Upon expression in HeLa cells, a protein consisting of Tm1–6 of CFTR followed by NBD1 and R was far more stable than a construct containing only Tm1–6 plus 20 additional cytosolic amino acids (Meacham *et al.*, 1999). Together with our results, these experiments support the conclusion that the NBD1 and R domains are sufficient to prevent access of Tm6 to the ER lumen and markedly increase the stability of CFTR.

A previous study in which the topology of CFTR was deduced from glycosylation at endogenous sites failed to detect assembly intermediates in which the NBD1 and R domains were exposed to the ER lumen (Chang *et al.*, 1994). It is possible that in mammalian cells such aberrant products are rapidly ubiquitinated and degraded, thus making their detection difficult. Alternatively, the translocation of entire cytosolic domains, as observed in the present study, might represent an extreme situation, and it is possible that normally only short sequence stretches including and following Tm6 may enter into the ER lumen. (The first endogenous consensus glycosylation site in NBD1 occurs 40 amino acids after the predicted C-terminus of Tm6.) Future studies will have to determine whether in the context of full-length CFTR Tm6 is indeed efficiently retained in the ER membrane during biogenesis or whether slippage of Tm6 into the ER lumen contributes to the inefficient maturation of the protein.

Since replacing two of the three basic residues in Tm6 with hydrophobic amino acids substantially inhibits translocation and increases the efficiency of proper membrane integration (Figure 3), why would evolution not have favoured a CFTR protein that has lost these potentially charged residues? The answer to this question is most likely tied to the critical functional role of these amino acids (Anderson *et al.*, 1991; Tabcharani *et al.*, 1993; Cotten and Welsh, 1999). In fact, several disease-causing mutations have been shown to occur in these basic residues (Dean *et al.*, 1990; Audrezet *et al.*, 1993; Sheppard *et al.*, 1993). It thus appears that the ion channel has been under strong evolutionary pressure to maintain a delicate balance between structural features required for function and for maturation.

Materials and methods

Fusion protein constructs

Constructs for expression in vivo. All molecular biology was performed using standard protocols (Sambrook *et al.*, 1989). The indicated segments of CFTR cDNA (Dr M.Drumm) were amplified using PCR. During amplification of some constructs, primers were used to introduce nucleotides encoding the amino acids ENLYFQ/GAMDP immediately following the CFTR fragment. The amino acids underlined represent a cleavage site for the TEV protease, in which proteolysis occurs between the Q and G residues (Carrington and Dougherty, 1988). CFTR constructs were cloned ahead of DNA encoding the enzymes invertase or Ura3 in the expression vector PYX 142 (Stratagene) to permit the constitutive expression of fusion proteins in yeast. A PCR-amplified fragment of CFTR encompassing Tm6 and 14 subsequent cytosolic amino acids of CFTR was cloned into the vector PSECY 306 between nucleotides encoding the carboxypeptidase Y signal sequence and invertase (Figure 6A). The TEV protease cDNA (Dr I.Moarefi) was amplified by PCR and inserted into the expression vector pYES2 (Invitrogen) to allow galactose-inducible expression of the protease in *S.cerevisiae*.

Constructs for expession in vitro. CFTR fragments for expression *in vitro* were cloned into the pcDNA3.1 myc–His_6 expression vector (Invitrogen) such that they contained an in-frame myc–His_6 tag at their extreme C-terminus. Where indicated, the N-terminal 256 amino acids of mature invertase were inserted between the CFTR sequences and the myc–His_6 tag. The codons CGG and AAA were mutated to CTG and ATA, respectively, using the Quick-change mutagenesis kit (Stratagene). These mutations enabled the production of proteins containing leucine in place of arginine 334 and isoleucine in place of lysine 335 in Tm6 of CFTR.

Analysis of CFTR fusion proteins in yeast

Expression. CFTR–invertase fusion proteins were expressed in the invertase-deficient yeast strains SEY6210 (Johnson *et al.*, 1987) and CKY406 (Dr C.Kaiser). Experiments involving the use of TEV protease utilized diploids generated from these two strains in order to achieve maximum galactose inducibilty of the protease. All procedures involving growth of yeast and introduction of vectors into cells have been described (Guthrie and Fink, 1991). Invertase activity assays were performed in order to evaluate the fractions of invertase and CFTR–invertase fusion protein that were secreted from yeast (Pelham *et al.*, 1988).

Preparation and analysis of cell lysates. Cell lysates for analysis by native PAGE were prepared by one of two methods. In experiments not evaluating cleavage by TEV protease, a 1 l yeast culture expressing CFTR–invertase fusions was grown at 30°C up to OD_{600} = 1. Cells were collected, washed in water and resuspended in 40 ml of ice cold buffer HS (0.15 M sodium acetate, 0.5 M potassium acetate, 1 mM dithiothreitol, 20 mM Tris pH 7.2, 20 mM EDTA). Cells were disrupted using a French Press at 25 kp.s.i. This lysate was centrifuged at 1000 *g* for 5 min to remove debris and unbroken cells. The supernatant was then layered over 20 ml of 1 M sucrose in buffer HS, and centrifuged for 1.5 h at 35 000 r.p.m. in a Beckman Ti 45 rotor. Pelleted material was resuspended in 5 ml of 30% sucrose in TBS (150 mM NaCl, 10 mM Tris pH 7.4) and stored in aliquots at –80°C. Experiments involving the co-expression of CFTR constructs with TEV protease were performed as follows. Several yeast colonies were resuspended in 3 ml of synthetic deficient medium containing 2% galactose and grown for 24 h at 30°C. This culture was added directly to 20 ml of the same medium and grown at 30°C for another 12–16 h. Ten OD_{600} units of these cells were washed once and resuspended in 100 µl of TBS. These cells were lysed by adding an approximately equal volume of glass beads and vortexing six times for 30 s each.

Purified membranes and glass-bead lysates were prepared for analysis as follows. Triton X-100 (TX-100) was added to the lysate at a final concentration of 2% followed by incubation for 15 min on ice. If the material was to be evaluated for the presence of glycosylation, the lysate was incubated with or without 20 000 U/ml of Endo H (New England Biolabs) overnight at 37°C. Proteolysis was achieved by adding proteinase K (Boehringer Mannheim) to a concentration of 100 µg/ml for 30 min on ice followed by addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM. Insoluble material was removed at 14 000 r.p.m. in a microcentrifuge for 5 min. The invertase activity in the supernatants was then analysed by a native gel assay exactly as described (Carlson *et al.*, 1981). Aliquots of 50 µl of bead-lysed cells were loaded in each lane. The amount of purified membranes used varied for different constructs and was empirically determined for each new preparation of membranes.

Analysis of CFTR fusion proteins upon in vitro translation

Translation and isolation of microsomes. *In vitro* translations were performed in the reticulocyte lysate translation system (Promega) supplemented with canine pancreatic microsomes and 100 µCi [^{35}S]methionine according to the manufacturer's instructions. After translation at 30°C, the reactions were diluted in TBS, layered on top of a 1 M sucrose cushion in TBS and microsomes pelleted in a Beckman TLA100 rotor by centrifugation for 30 min at 50 000 r.p.m. (4°C).

Immunoprecipitation. The pelleted material was solubilized in 100 µl of TBS containing 1% TX-100 for 30 min at 4°C. The lysate was clarified by centrifugation in a microcentrifuge at 14 000 r.p.m. for 5 min. The supernatant was incubated with 1 µg of the anti-myc antibody 9E10 or a non-relevant rabbit serum for 30 min on ice. Immune complexes were isolated by adding 10 µl of packed protein G–Sepharose beads and incubating for an additional 30 min. The beads were washed four times with the solubilization buffer. N-linked glycans were removed from the isolated proteins by incubation of the washed beads with 100 U of PNGase F (New England Biolabs) for 2 h at 37°C.

Proteinase K and PNGase F assays. To evaluate the protease accessibility of proteins, 5 µl translation/translocation reactions were incubated with 100 µg/ml proteinase K on ice for 30 min. Pefabloc was added to 1 mM to stop proteolysis.

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