FKBP38 Peptidylprolyl Isomerase Promotes the Folding of Cystic Fibrosis Transmembrane Conductance Regulator in the Endoplasmic Reticulum*

Received for publication, June 10, 2011, and in revised form, October 24, 2011 Published, JBC Papers in Press, October 26, 2011, DOI 10.1074/jbc.M111.269993

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Background: FKBP38 regulates the biogenesis of plasma membrane ion channels.

Results: FKBP38 inhibits protein synthesis through its membrane anchorage and promotes CFTR post-translational folding through its PPIase domain, both negatively regulated by Hsp90 through the tetratricopeptide repeat domain.

Conclusion: FKBP38 PPIase plays an important role in CFTR biogenesis.

Significance: Our findings demonstrate an independent contribution of FKBP38 to CFTR biogenesis.

FK506-binding protein 38 (FKBP38), a membrane-anchored, tetratricopeptide repeat (TPR)-containing immunophilin, associates with nascent plasma membrane ion channels in the endoplasmic reticulum (ER). It promotes the maturation of the human ether-à-go-go-related gene (HERG) potassium channel and maintains the steady state level of the cystic fibrosis transmembrane conductance regulator (CFTR), but the underlying mechanisms remain unclear. Using a combination of steady state and pulse-chase analyses, we show that FKBP38 knockdown increases protein synthesis but inhibits the post-translational folding of CFTR, leading to reduced steady state levels of CFTR in the ER, decreased processing, and impaired cell surface functional expression in Calu-3 human airway epithelial cells. The membrane anchorage of FKBP38 is necessary for the inhibition of protein synthesis but not for CFTR post-translational folding. In contrast, the peptidylprolyl cis/trans isomerase active site is utilized to promote CFTR post-translational folding but is not important for regulation of protein synthesis. Uncoupling FKBP38 from Hsp90 by substituting a conserved lysine in the TPR domain modestly enhances CFTR maturation and further reduces its synthesis. Removing the N-terminal glutamate-rich domain (ERD) slightly enhances CFTR synthesis but reduces its maturation, suggesting that the ERD contributes to FKBP38 biological activities. Our data support a dual role for FKBP38 in regulating CFTR synthesis and post-translational folding. In contrast to earlier prediction but consistent with in vitro enzymological studies, FKBP38 peptidylprolyl cis/trans isomerase plays an important role in membrane protein biogenesis on the cytoplasmic side of the ER membrane, whose activity is negatively regulated by Hsp90 through the TPR domain.

FK506-binding proteins (FKBPs) constitute a family of proteins that catalyze the cis/trans interconversion of the peptide bond preceding the proline residue. The prolyl isomerization is important for both de novo folding of nascent polypeptide chains and the regulation of activities of mature client proteins (1). FKBP38 is a unique member of the FKBP family (2) in that it is membrane-anchored (3), and its peptidylprolyl cis/trans isomerase (PPIase) activity is regulated by calmodulin (4). FKBP38 has been localized to the membranes of both the mitochondria and the endoplasmic reticulum (ER) (3, 5, 6).

Although FKBP38 plays a great variety of roles in cell regulation (7) including cell size regulation (8), apoptosis (3), development of neural tubes (9), mammalian target of rapamycin (mTOR) signaling (10), hypoxia response (11), and viral replication (12), it associates with nascent plasma membrane ion channel proteins in the ER and regulates their biogenesis (13, 14). In HEK293 cells, FKBP38 was shown to promote the processing of the voltage-dependent delayed rectifier potassium channel known as the human ether-à-go-go-related gene (HERG) product that is responsible for the long QT syndrome (13). In the same cell line, FKBP38 was demonstrated to have a greater impact on the steady state level of the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel responsible for cystic fibrosis (14). Interestingly, in both cases FKBP38 was found to associate with both ion channels in the ER together with Hsp70, Hsp90, Hsp40, Hop, and p23 (13, 14).

An Hsp70-Hsp90 chaperone relay system is well studied in the context of the conformational activation of steroid recep-

2 The abbreviations used are: FKBP, FK506-binding protein; CFTR, cystic fibrosis transmembrane conductance regulator; DM-CHX, N-(N-[(N,N,N-Trimethylammonium)methyl]cyclohexylmethyl)cycloneheximide; ER, endoplasmic reticulum; ERAD, ER-associated degradation; HERG, human ether-à-go-go-related gene; PPIase, peptidylprolyl cis/trans isomerase; SR, steroid receptor; TPR, tetratricopeptide repeat; mTOR, mammalian target of rapamycin; SPO, 6-methoxy-N-[3-(sulfopropyl)quinolinium; Cntrl, control; rk308A, shRNA-refractory K308A mutant FKBP38; ERD, glutamate-rich domain; TM, transmembrane domain; ALLN, N-acetyl-i-leucinyl-i-leucinyl-i-norleucine; EGFP, enhanced GFP.
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tors (SRs) (15). In such a system, multiple co-chaperones coordinate the sequential recruitment of Hsp70 and Hsp90, leading to a conformational change in SR, which is required for its hormone binding (15, 16). FKBP52, a tetratricopeptide repeat (TPR)-containing immunophilin, binds Hsp90 through its TPR domain. Although it is not required for SR-Hsp90 heterocomplex assembly or SR conformational activation, FKBP52 is involved in the regulation of SR trafficking between the cytoplasm and the nucleus (17–20). The fact that Hsp90 plays an important role in the maturation of both CFTR (21) and the HERG channel (22) and the association of both ion channels with Hsp70 in the ER (13, 14) raise the possibility that a similar Hsp70-Hsp90 chaperone system might facilitate conformational maturation of the nascent ion channels as well. Because FKBP38 shows a high degree of similarity to FKBP52, an enticing hypothesis has been proposed where FKBP38 plays an analogous role in the maturation and the ER-to-Golgi trafficking of CFTR or the HERG channel (13, 14, 22).

However, given that molecular chaperones have significant impact on multiple aspects of ion channel biogenesis including co-translational folding, post-translational maturation, ER quality control, and ER-associated degradation (ERAD) (13, 14, 21–34), before such a hypothesis can be tested directly a careful characterization of the FKBP38 role in membrane protein biogenesis needs to be conducted, and its functional relationship with Hsp90 must be defined. To this end we performed a systematic functional dissection of FKBP38 in the biogenesis of CFTR. Our data indicate that FKBP38 regulates both the synthesis and post-translational folding of CFTR. Its membrane anchorage is not necessary for the regulation of CFTR post-translational folding but is required for the regulation of protein synthesis. Its PPlase is important for CFTR post-translational folding but not for CFTR synthesis. Strikingly, the interaction of FKBP38 with Hsp90 through the TPR domain negatively impacts FKBP38 regulation of both CFTR synthesis and its post-translational folding. Our data highlight a pro-folding effect for FKBP38 in CFTR biogenesis mediated largely through its PPlase domain.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—The CFTR monoclonal antibodies (mAbs) used include MM13-4, M3A7, and 13-1 (Millipore, Temecula, CA). FKBP38 antibodies include rabbit polyclonal antibody FK38N1 (from Dr. Michiko Shirane at Kyushu University, Fukuoka, Japan), a mouse monoclonal antibody recognizing amino acids 1–325 of human FKBP38 (R&D Systems, Santa Cruz, CA). Other antibodies used include anti-Hdj-2 mouse mAb (Abcam, Cambridge, MA), anti-Hsp105 mAB (Novocastra, Newcastle upon Tyne, U.K), anti-Hsc70, anti-Hsp90α, and anti-Hsp90β mAbs (Stressgen, Ann Arbor, MI), anti-calnexin and anti-Hsp70 polyclonal antibodies (Stressgen), anti-actin mAb (Millipore), and anti-glutathione S-transferase (GST) goat polyclonal antibodies (GE Healthcare).

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA), and Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA). The FKBP38 PPlase inhibitor N-(N’N’-dimethylcarboxamidomethyl)cycloheximide (DM-CHX) was synthesized according to a published procedure (35). ALLN was purchased from Sigma. Protein G-Sepharose 4 Fast Flow was purchased from GE Healthcare or East Coast Bio (North Berwick, ME). Glutathione-Sepharose 4B was purchased from GE Healthcare.

Plasmid Construction—The FKBP38 shRNA expression plasmid was constructed using IMG-800–1 vector (Imgenex, San Diego, CA) according to the manufacturer’s protocol. The control plasmid contains a non-targeting sequence 5’-TCA GTC ACG TTA ATG GTC GTT-3’, and the FKBP38 target sequence used is 5’-GGA GTG CTT GAA CAT CTT-3’.

The full-length FKBP38 coding sequence was amplified from Human Adult 8 Tissue Gene Pools cDNA (NT Omics, San Mateo, CA) by polymerase chain reaction (PCR). An Nhel restriction site was added to the 5’ end and a KpnI site to the 3’ end of the coding sequence by appending to the 5’ end of the primers. The amplified fragment was inserted into the Nhel and KpnI sites of pcDNA3.1(+) to generate the full-length FKBP38 expression plasmid pcDNA3.1(+)–FKBP38. FKBP38 mutant expression constructs were built upon the pcDNA3.1(+)–FKBP38 by overlapping PCR using the Nhel-KpnI cassette. The ΔERD mutant lacks amino acid residues 2–92, the ΔTM mutant lacks amino acid residues 390–413, and the ΔPPlase mutant lacks amino acid residues 120–205.

The shRNA-refractory FKBP38 expression plasmid pcDNA3.1(+)–rFKBP38 was constructed by introducing silent mutations to the shRNA target sequence within the pcDNA3.1(+)–FKBP38 plasmid to yield 5’-GGA GTG CTT GAA CAT CTT-3’. The silent mutations were introduced by overlapping PCR, and the DNA fragment was ligated back into the Nhel-KpnI cassette of the same plasmid. The shRNA-refractory mutant FKBP38 plasmids such as refractory K308A mutant FKBP38 (rK308A), rΔERD, rΔPPlase, and rΔTM were generated likewise.

To construct the pcDNA3.1(+)–GST plasmid, the GST coding sequence was amplified from the pET41a(+) vector (Novagen, Madison, WI) by PCR. Nhel and Xhol sites were added to the 5’ and 3’ ends of the amplified fragment, respectively. The fragment was then inserted between the same sites of pcDNA3.1(+). To generate the GST-tagged FKBP38 expression plasmid, the FKBP38 coding sequence covering the first 230 amino acids was first amplified from Human Adult 8 Tissue Gene Pools cDNA by PCR and inserted in-frame after GST into the pET41a(+) vector to yield pET41a(+)–GST-FKBP38–(1–230). Then the GST-FKBP38 N-terminal fragment was amplified by PCR from the pET41a(+)–GST-FKBP38–(1–230) plasmid. An Nhel site was added to the 5’ end of the PCR product by appending to the 5’ end of the primer. The derived fragment was inserted into the pcDNA3.1(–)–FKBP38 between the Nhel and an endogenous Xhol site at nucleotide 62 of the FKBP38 coding sequence to generate the pcDNA3.1(–)–GST–FKBP38 expression plasmid. GST-tagged FKBP38 mutants such as K308A and R312A were generated by overlapping PCR using the Nhel-KpnI cassette. PCR-derived regions of all plasmid constructs were confirmed by DNA sequencing.

The pcDNA3-EGFP plasmid was obtained from Addgene (Cambridge, MA). The EGFP coding sequence was excised...
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Quantitative Affinity Precipitation of GST-FKBP38 Fusion Proteins—HEK293 cells were transfected with various forms of GST-FKBP38 fusion constructs. Twenty-four hours post-transfection, cells were lysed in Triton X-100 lysis buffer supplemented with the Complete™ protease inhibitor mixture. Equal proportions of cell lysates were incubated with glutathione–Sepharose 4B overnight. The bound proteins were eluted in 1% SDS solution and resolved on SDS-PAGE. The associated chaperones were detected by quantitative immunoblotting. HEK293 cells transfected with GST alone served as the negative control. The level of the chaperones associated with various forms of GST-FKBP38 fusion proteins were first subtracted by the level of the same chaperones associated with the GST control and then normalized to the level of the corresponding GST-FKBP38 fusion proteins as detected by GST antibody.

Fluorescence-based Iodide Efflux Assay—The cAMP-dependent iodide efflux was measured using fluorescent dye 6-methoxy-N-(3-sulfopropyl)-quinolinium (SPQ) (TEF Labs, Austin, TX) largely as described by Munkonge et al. (37). Calu-3 cells stably expressing the control or FKBP38 shRNA were grown on glass coverslips overnight. The cells were loaded with SPQ by hypotonic shock and inserted into a perfusion chamber with a temperature control at 37 °C. Live cell imaging was performed using a Polychrome IV monochrometer-based fluorescence imaging system coupled to a Nikon TE 2000 microscope as previously described (38). SPQ fluorescence was excited at 360 nm using dichroic mirror (FF409) and collected using Semrock brightline emission filter passing 447 ± 30-nm light. The cells were perfused with NaI solution to attain maximal quenching of the SPQ fluorescence. Then cAMP-dependent iodide efflux was stimulated by perfusion with 10 μM forskolin plus 100 μM isobutylmethylxanthine in NaNO₃ solution. The fluorescence intensity (F₀) was measured at 30-s intervals and normalized to the value at the maximal quenching (Fₚ). Results were typically derived from ~20 cells per field. Multiple fields were recorded for the same or different coverslips. The initial rates were estimated by linear fits to the first 60 s of the cAMP-stimulated iodide efflux.

RESULTS

FKBP38 Inhibits CFTR Synthesis, Stabilizes It in the ER, and Improves Its Processing—To analyze the role of FKBP38 in CFTR biogenesis, we generated a HEK293 cell line stably expressing FKBP38 shRNA (F38i). HEK293 cells stably expressing a non-targeting shRNA (Cntrl) served as the control. We transiently expressed wild-type CFTR into the F38i and the Cntrl cell lines and found that the steady state level of CFTR is reduced by 59% in the immature, ER-localized, core-glycosylated form (band C) (Fig. 1A) and by 53% in the mature, Golgi-processed, complex-glycosylated form (band B) and by 53% in the mature, core-glycosylated form (band A) (Fig. 1A) (Fig. 1A). In the F38i cells, the FKBP38 level is reduced by 97%, with no significant change in the levels of Hsc70, Hsp70, Hsp90α, or Hsp105 (Fig. 1B).

To probe the specific roles of FKBP38 in the synthesis, maturation, and ERAD of CFTR, we performed pulse-chase analysis on the F38i and Cntrl cell lines transiently expressing wild-type CFTR. In the F38i cells, the level of radiolabeled CFTR after the 30-min pulse is 192% greater than in the Cntrl cell line.

from the plasmid by Xhol and Xbal sites and inserted into pcDNA3.1(+) vector using the same sites to generate the pcDNA3.1(+)–EGFP plasmid. To construct the pcDNA3.1(−)–GST–EGFP plasmid, the EGFP coding sequence was amplified from pcDNA3.1(+)–EGFP using PCR. HindIII and Xhol sites were added to the N and C termini of EGFP coding sequence through the upper and lower primers, respectively. The fragment was then cloned into pET41a(+) vector between the same sites so that the EGFP was in-frame with the GST and the intervening coding sequences in the vector. Then the complete GST–EGFP coding sequence was subcloned into pcDNA3.1(+) using the Xbal and Xhol sites. The CFTR expression plasmids pcDNA3.1(+)–CFTR–WT and pcDNA3.1(+)–CFTR–ΔF508 have been described previously (36).

Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml each of penicillin and streptomycin. Calu-3 cells were maintained in minimum essential medium Eagle with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS, and 100 units/ml each of penicillin and streptomycin. Cell transfection was performed using Lipofectamine 2000 (Invitrogen). To prepare the FKBP38 knockdown stable cell line (F38i), HEK293 cells were transfected with the FKBP38 shRNA construct, and the cells were subjected to clonal selection by 400 μg/ml G418. HEK293 cells stably expressing the non-targeting shRNA construct were generated in a similar manner. Calu-3 cells stably expressing FKBP38 shRNA or the non-targeting shRNA were generated in a similar fashion.

Cell Lysis and Quantitative Immunoblotting—Cells were lysed on ice with Triton X-100 lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 (v/v), and the Complete™ protease inhibitor mixture (Roche Diagnostics) for 30 min. Insoluble material was then removed by centrifuging at 16,000 × g for 20 min. Protein concentration was determined using the Bradford assay. Cell lysates were separated by SDS-PAGE, immunoblotted with relevant antibodies, and visualized by ECL. Protein loading was adjusted according to its intracellular abundance. Multiple exposures were taken to make sure that the intensity of bands is within the dynamic range. The protein bands were quantified by using ImageJ software (National Institutes of Health).

Pulse-Chase Analysis—Equal numbers of cells were seeded in 60-mm cell culture dishes. The cells were transfected with CFTR alone or in combination with other expression plasmids, and total amount of plasmids used was kept even among different dishes. After incubating the cells at 37 °C for 16–24 h post-transfection, cells were starved in cysteine- and methionine-free medium for 30 min, pulse-labeled with EasyTag Express 35S protein labeling mix (PerkinElmer Life Sciences) for 30 min, and chased for the indicated times with complete medium. Cells were lysed as described above, and CFTR was recovered by immunoprecipitation. The radiolabeled CFTR were visualized by autoradiography and quantified by phosphorimaging using Typhoon FLA7000 Imager (GE Healthcare) with Image Quant software.
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In contrast, the rate of disappearance of CFTR band B in the F38i cells during the chase period is much greater than that in the Cntrl cells (Fig. 1C). These results suggest that FKBP38 depletion increases both CFTR synthesis and its post-translational ERAD. We have also observed a modest decrease in the average rate of conversion from CFTR band B to band C during the chase as a result of FKBP38 knockdown (Fig. 1C). When referring to the steady state levels of CFTR in the two cell lines (Fig. 1A), it is obvious that the increase in post-translational ERAD of CFTR had overpowered the increase in CFTR synthesis in the F38i cells, leading to reduced steady state levels of CFTR in both band B and band C.

To confirm that the above differences were not caused by the inherent differences between the F38i and Cntrl cell lines, we introduced a shRNA-refractory version of wild-type FKBP38 (rF38) back into the F38i cell line and repeated the pulse-chase analysis. We found that FKBP38 rescue reduces CFTR synthesis by 38%, decreases the rate of its turnover in the ER, and modestly increases the rate of CFTR maturation to band C (Fig. 1D). Therefore, we conclude that FKBP38 reduces the synthesis of CFTR and enhances the post-translational folding of CFTR.

R312A and K308A Substitutions Interfere with FKBP38 Association with Hsp90 within the Cell—FKBP38 harbors a TPR domain consisting of three TPR motifs proximal to its TM region (Fig. 2A). This TPR domain shares high sequence similarity to the Hsp90 binding TPR domains of other TPR proteins such as FKBP52, PP5, and Cyp40. FKBP38 has been shown to bind the C-terminal domain of Hsp90 in a Ca\(^{2+}\)/calmodulin-dependent manner (39). Two highly conserved basic residues within TPR3 of PP5 are required for its TPR-dependent interaction with Hsp90 (40). We performed amino acid sequence alignment on the TPR3s of FKBP38, PP5, FKBP52, and Cyp40 and found that indeed the two basic residues are conserved among the four TPR proteins. In FKBP38, Lys-308 and Arg-312 correspond to the two conserved basic residues seen in PP5 (Fig. 2A). We substituted these two residues individually by alanine and expressed the GST-tagged mutant FKBP38 in HEK293 cells. We found that both R312A and K308A substitutions reduce the association of FKBP38 with Hsp90, with the latter being more effective, and this is true for both isoforms of Hsp90 (Fig. 2B).

Uncoupling FKBP38 from Hsp90 Further Reduces CFTR Synthesis and Promotes CFTR Maturation—To test the functional consequence of uncoupling FKBP38 from Hsp90, we introduced the shRNA-refractory version of K308A mutant (rK308A) into the F38i cells and found that rK308A, compared with rF38, reduces the synthesis of CFTR and modestly enhances its maturation (Fig. 2D). Therefore, uncoupling FKBP38 from

**FIGURE 1. FKBP38 reduces the synthesis of CFTR and promotes its post-translational folding in the ER.** A and B, HEK293 cells stably expressing a non-targeting shRNA (Cntrl) or FKBP38 shRNA (F38i) were transfected with wild-type CFTR. After 24 h, cells were lysed, and equal amounts of lysates were immunoblotted for the indicated proteins. The protein levels were quantified by densitometry. All values were normalized to actin and then to the values of the Cntrl to facilitate comparison. The means and S.E. are shown. An unpaired, two-tailed t test was performed for all experiments, n = 3. Where indicated, * and ** denote p ≤ 0.05 and 0.01, respectively. C, the Cntrl and F38i cells were transfected with wild-type CFTR and subjected to pulse-chase analysis. D, the F38i cells were cotransfected with wild-type CFTR together with EGFP (Cntrl) or the shRNA-refractory FKBP38 expression plasmid (rF38) and subjected to pulse-chase analysis. For all pulse-chase analyses, the numbers below the autoradiograph indicate the relative levels of CFTR band B at the 0-h chase. To facilitate comparison of the rates of band B turnover and the rates of band C appearance, the levels of CFTR in bands B and C at all time points were normalized to the level of band B at the 0-h chase in each set. The means and S.E. for two independent experiments are shown.
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Hsp90 enhances FKBP38 wild-type activities in both CFTR synthesis and post-translational folding. This result suggests that Hsp90 binding to FKBP38 modestly inhibits FKBP38 activities in the context of CFTR biogenesis.

FKBP38 Inhibits Protein Synthesis without Affecting Co-translational ERAD—To explore the mechanism underlying FKBP38 inhibition of CFTR synthesis, we introduced a cytosolic control protein, GFP-GST fusion protein, into the F38i cells, and assessed its rate of synthesis by a pulse-labeling time course. Compared with the no-rescue control (Cntrl), expression of rF38 leads to a specific decrease in the rate of GFP-GST synthesis (Fig. 3A), suggesting that FKBP38 also inhibits the synthesis of cytoplasmic proteins.

The reduction in the level of radiolabeled CFTR after a 30-min pulse due to rF38 rescue (Fig. 1D) could be attributable to increased co-translational ERAD of CFTR. To test this possibility, we conducted pulse-labeling in the absence and presence of proteasome inhibitor ALLN and observed a proportional increase in CFTR labeling among the Cntrl, rF38, and rK308A as a result of ALLN treatment (Fig. 3B). Therefore, co-translational ERAD is unlikely to contribute to the reduction in the level of CFTR after 30-min pulse.

FKBP38 Membrane Anchorage Is Necessary for Regulating Protein Synthesis but Not for Post-translational Folding of CFTR—FKBP38 is unique among TPR immunophilins in that it is membrane-anchored. To explore the functional significance of its membrane anchorage, we deleted its transmembrane domain and reintroduced the shRNA-refractory version of the mutant into the F38i cells. Pulse-chase analysis revealed that removing the TM domain of FKBP38 abolishes its inhibition of CFTR synthesis but does not impact its role in the post-translational folding of CFTR. This is reflected in a 31% increase in the level of the radiolabeled CFTR after pulse as compared with rF38 and in an unchanged rate of CFTR band B turnover or band C appearance during the chase period (Fig. 4A). Consistent with the pulse-chase analysis, the steady state levels of CFTR in both band B and band C are increased when the rαTM mutant was introduced as compared with the rF38 control (Fig. 4B). Again, the observed change in CFTR synthesis is not attributable to co-translational ERAD as demonstrated by pulse analysis in the presence and absence of ALLN (Fig. 4C).

FKBP38 Glutamate-rich Domain (ERD) Contributes Mostly to Both the Synthesis and Post-translational Folding of CFTR—The functional role of the N-terminal ERD of FKBP38 is not fully understood. We tested the role FKBP38 ERD in CFTR biogenesis by rescuing the F38i cells with the rΔERD mutant. Compared with the rF38, introduction of rΔERD
slightly increases the steady state level of CFTR in both band B and band C (Fig. 5A). Consistent with this, pulse-chase analysis revealed a modest increase in CFTR synthesis and a slight decrease in its rate of maturation (Fig. 5B). Therefore, removal of the ERD from FKBP38 has only a mild negative impact on the two activities of FKBP38 in CFTR biogenesis.

FKBP38 PPIase Site Is Important for Promoting CFTR Post-translational Folding—To test the role of the PPIase domain of FKBP38, we constructed an shRNA-refractory PPIase domain deletion mutant (rΔH9004 PPIase) and introduced it into the F38i cells. Based on pulse-chase analyses, deletion of the PPIase domain dramatically reduces the maturation efficiency of CFTR and slightly destabilizes CFTR band B but has minimal impact on CFTR synthesis (Fig. 6A), suggesting that the PPIase domain of FKBP38 is important for post-translational folding of CFTR but not for the regulation of protein synthesis. Consistent with this, we have observed a decrease in the steady state levels of both CFTR band B and band C (Fig. 6B). As all the FKBP38 antibodies we used recognize a region of FKBP38 that includes the PPIase domain, the intensity of the band corresponding to the rΔPPIase protein as detected by immunoblotting does not reflect its actual protein abundance (Fig. 6B, asterisk).

To further test if the above effect is produced by the lack of the PPIase domain or specifically by the absence of the PPIase activity, we treated both the control and F38i HEK cells with DM-CHX, a FKBP38-selective PPIase inhibitor (35). Pulse-chase analyses revealed a reduction in CFTR maturation but not in CFTR synthesis in the control cell line (Fig. 7A). Such an effect on CFTR maturation is largely absent in the F38i cell line where 97% of FKBP38 has been removed (Fig. 7B), suggesting that the observed effect of DM-CHX is specific for FKBP38. Together, the above data reveal the importance of a freely accessible PPIase site in the FKBP38-mediated post-translational folding of CFTR.

FKBP38 Regulates the Cell Surface Functional Expression of CFTR in Calu-3 Human Airway Epithelial Cells—To assess the physiological consequence of FKBP38 knockdown in airway epithelial cells expressing endogenous CFTR, we generated a Calu-3 cell line stably expressing endogenous CFTR. Knockdown of FKBP38 in Calu-3 cells results in a decrease in CFTR band B by 76% and a decrease in CFTR band C by 51%
To assess the impact on CFTR cell surface functional expression, we performed iodide efflux assay on the two cell lines and observed a dramatic decrease (by 64%) in the rate of the cAMP-stimulated iodide efflux (Fig. 8, B and C). These data suggest that FKBP38 does have a major impact on the CFTR-mediated chloride conductance in airway epithelial cells. To further test the impact of FKBP38 PPIase site on CFTR biogenesis in Calu-3 cells, we treated both the Cntrl and F38i Calu-3 cells with DM-CHX with increasing duration and observed a time-dependent reduction in the steady state level of CFTR in band B and band C in the Cntrl Calu-3 cells but not the F38i Calu-3 cells (Fig. 8D). A lighter exposure was used to quantify the levels of CFTR band C to avoid saturation (Fig. 8D). The time-dependent decrease in band C is greater than that in band B, consistent with a role for FKBP38 PPIase in CFTR maturation in Calu-3 cells (Fig. 8D). Taken together, these data suggest that FKBP38 regulates CFTR cell surface functional expression in a PPIase site-dependent fashion in human airway epithelial cells.

DISCUSSION

Early data on the FKBP38 role in the biogenesis of CFTR and the HERG channel have largely relied on the steady state level of the ER-localized, core-glycosylated form and the Golgi-processed complex glycoform (13, 14). In the current study we combined steady state and pulse-chase analyses and revealed a dual role for FKBP38 in regulating the synthesis and post-translational folding of CFTR (Figs. 1 and 3B). In HEK293 cells the inhibition of synthesis and promotion of post-translational folding oppose each other in the control of the steady state level of CFTR both in the ER and at the cell periphery, leading to reduction in the steady state levels of both band B and band C (Fig. 1). Interestingly, the inhibition of synthesis is not restricted to CFTR but also includes a cytoplasmic protein (Fig. 3A), suggesting that FKBP38 is likely to inhibit general protein translation. This role of FKBP38 requires membrane anchorage (Fig. 4) but not its PPIase activity or PPIase domain (Figs. 6 and 7). A role for FKBP38 in regulating protein translation might be mediated through its inhibition of mTOR complex 1 (10, 41), although the cellular context and the mechanism of such inhibition remain to be fully elucidated (41–47).

We have clearly shown that FKBP38 curbs post-translational ERAD and improves maturation of CFTR (Fig. 1). Although
reducing ERAD can improve CFTR maturation (Fig. 1D). FKBP38 PPIase appears to have a greater impact on CFTR maturation than its post-translational ERAD (Figs. 6 and 7). Moreover, uncoupling FKBP38 from Hsp90 by K308A substitution improves maturation but has little impact on the post-transla-
tional ERAD of CFTR (Fig. 2D). These data suggest that the regulation of CFTR post-translational ERAD by FKBP38 might be mediated through a separate mechanism. FKBP38 was found to anchor the 26S proteasome to organellar membrane through its TPR domain (48). However, whether this TPR-dependent anchorage plays a role in stabilizing CFTR from ERAD remains to be determined. Alternatively, FKBP38 might stabilize CFTR through a PPIase site-mediated chaperone activity (49). FKBP38 was shown to protect citrate synthase from aggregation in vitro and stabilize Bcl-2 from degradation in the cell (50). A recent study clearly demonstrated that FKBP38 stabilizes Bcl-2 post-translationally by binding to it (51). Therefore, FKBP38 may stabilize CFTR by direct protein-protein interaction. As deletion of the PPIase domain of FKBP38 only slightly reduces CFTR stability in the ER (Fig. 6A), other domains or additional mechanisms might be important for CFTR stabilization.

TPR immunophilin FKBP52 is a component of the chaperone network responsible for the Hsp90-dependent activation of SRs (15, 16). FKBP52 binds to Hsp90 through its TPR domain and regulates the localization of SRs (17–20). We have not identified an analogous role for FKBP38 in the Hsp90-dependent localization of CFTR. Instead, we found that disruption of FKBP38-Hsp90 interaction by K308A substitution in TPR3 further enhances FKBP38 activity in inhibiting protein synthesis and in promoting CFTR maturation (Fig. 2), suggesting that Hsp90 binding inhibits FKBP38 cellular function. This is consistent with in vitro studies demonstrating that Hsp90 is a negative regulator of FKBP38 enzymatic activity (39). Nevertheless, the role of FKBP38 in inhibiting post-translational ERAD of CFTR is not affected by uncoupling Hsp90 from FKBP38 (Fig. 2), suggesting that Hsp90 has limited impact on the post-translational ERAD of CFTR through FKBP38.

FKBP52 has a constitutively active PPIase domain, but its PPIase domain rather than its PPIase activity is required for the potentiation of the hormone-dependent transactivation of SRs (52). In contrast, the PPIase of FKBP38 is induced by calmodulin (4) and inhibited by Hsp90 (39). A recent publication demonstrates by crystal structure analysis that PPIase inhibitor DM-CHX directly binds in a substrate-like manner to the isomerase site of a FKBP domain, underlining that it can directly compete with proline residues of protein substrates for the active site of FKBP52 (53). Treatment of cells with DM-CHX reduces the post-translational maturation of CFTR (Figs. 7 and 8D), suggesting that DM-CHX displaces a native substrate, probably CFTR, from the active site of FKBP38. Given that prolyl isomerization is involved in de novo folding of nascent polypeptide chains (1), it is highly likely that FKBP38 PPIase facilitates prolyl isomerization of nascent CFTR on the cytoplasmic side of the ER membrane and, hence, promotes CFTR folding and maturation. However, direct evidence for nascent CFTR as a substrate for FKBP38 PPIase is currently missing.

Presently there is no method available in the literature (i) to identify the “hot” proline residue among the 45 prolines of CFTR or (ii) to measure a catalyzed prolyl isomerization in such a large protein. As PPIase is also known to regulate the activities of mature client proteins (1), although unlikely, we cannot
exclude the possibility that FKBP38 PPIase influences CFTR folding by regulating the activity of another protein.

One of the most prominent features of FKBP38 is its membrane anchorage, and this is highly suggestive of a role for FKBP38 in recruiting cytoplasmic chaperones to the ER membrane. Surprisingly, deletion of the TM domain of FKBP38 has little impact on the post-translational folding of CFTR but abolishes FKBP38 inhibition of protein synthesis (Fig. 4). These results are consistent with an important role for FKBP38 PPIase site in CFTR post-translational folding and further suggest that membrane anchorage of FKBP38 might be essential for its regulation of protein synthesis. Recently, FKBP38 was reported to interact with the hypoxia-inducible factor prolyl-4-hydroxylase domain 2 protein and regulate its stability in a PPIase-independent manner (11). Such a stabilization effect was found to depend on the membrane anchorage of FKBP38 (54). Therefore, although the membrane anchorage of FKBP38 is important for some of its cellular roles, it is not essential for the post-translational folding of CFTR.

A less well characterized domain in FKBP38 is its N-terminal ERD. This domain was truncated in the early studies of FKBP38 as a result of the usage of a downstream start codon (2). In both HEK293 and Calu-3 cells, the endogenous FKBP38 is the long form. We tested the role of the ERD in FKBP38-regulated CFTR biogenesis and found that the ERD contributes modestly to both inhibition of protein synthesis and CFTR post-translational folding (Fig. 5). Interestingly, deletion of an N-terminal region including the ERD was found to abolish FKBP38 association with prolyl-4-hydroxylase domain 2 (PHD2) (11), suggesting that the ERD might play a role in FKBP38-mediated stabilization of PHD2.

As most of our study was conducted on HEK293 cells expressing exogenous CFTR, we verified the role of FKBP38 in CFTR biogenesis in Calu-3 human airway epithelial cells expressing endogenous CFTR and found that indeed FKBP38 regulates the CFTR-mediated chloride conductance at the plasma membrane in a FKBP38 active site-dependent manner (Fig. 8).

Based upon our data, we propose a working model for FKBP38 regulation of CFTR biogenesis (Fig. 9). FKBP38 plays a major role in the regulation of post-translational folding of CFTR through its PPIase site, the blockade of which by DM-CHX abolishes assistance in post-translational folding. Such regulation does not require membrane anchorage. In addition, FKBP38 also indirectly regulates general protein synthesis possibly through mTOR complex 1 signaling or a still unknown mechanism. The latter regulation requires membrane anchorage. Regulation at both levels is inhibited by Hsp90 binding to FKBP38 through the TPR domain. We believe that such regulation of CFTR folding is apart from the Hsp90-dependent regulation of CFTR maturation and stabilization in the ER (14, 21). FKBP38-mediated post-translational folding of CFTR might play an ancillary role in the overall chaperone-mediated post-translational folding of CFTR. However, at the present time we cannot rule out a possible cross-talk between the two types of regulation of CFTR post-translational folding involving Hsp90.

Looking beyond the pro-folding effect of FKBP38 in ion channel biogenesis, this immunophilin plays critical roles in many aspects of cell signaling, especially in regulation of apoptosis through Bcl-2 (7). One of the major roles for FKBP38 in the regulation of apoptosis is through stabilizing Bcl-2 from degradation. In this context, FKBP38 functions as a stabilizing binding partner for Bcl-2, conferring longer half-life and, therefore, higher steady state level of the anti-apoptotic molecule (51). This is analogous to FKBP38 inhibition of the post-translational ERAD of CFTR (Fig. 1), although the specific nature of the FKBP38-client interaction might be different. A better understanding of the basic biology of the FKBP38-facilitated protein folding within the cell will provide critical insights into FKBP38 roles in cell signaling.

Acknowledgments—We appreciate the gift of anti-FKBP38 rabbit antiserum FK38N1 from Dr. Michiko Shirane (Kyushu University). We thank Dr. William E. Balch (The Scripps Research Institute, La Jolla, CA) for providing CFTR plasmid and cell lines. We thank Gargi Roy for making the pcDNA3.1(+) -GST construct.

REFERENCES

1. Edlich, F., and Fischer, G. (2006) Handb. Exp. Pharmacol. 172, 359–404
2. Nielsen, J. V., Mitchemore, C., Pedersen, K. M., Kjaerulff, K. M., Finsen, B., and Jensen, N. A. (2004) Genomics 83, 181–192
3. Shirane, M., and Nakayama, K. I. (2003) Nat. Cell Biol. 5, 28–37
4. Edlich, F., Weiwad, M., Erdmann, F., Fanghanel, J., Jarczowski, F., Rahfeld, J. U., and Fischer, G. (2005) EMBO J. 24, 2688–2699
5. Germain, M., and Shore, G. C. (2003) Sci STKE 2003, pe10
6. Wang, H. Q., Nakaya, Y., Du, Z., Yamane, T., Shirane, M., Kudo, T., Takoda, M., Takebayashi, K., Noda, Y., Nakayama, K. I., and Nishimura, M. (2005) Hum. Mol. Genet. 14, 1889–1902
7. Edlich, F., and Lücke, C. (2011) Curr. Opin. Pharmacol. 11, 348–353
8. Rosner, M., Hofer, K., Kubista, M., and Hengstschläger, M. (2003) Oncogene 22, 4786–4798
9. Bulgakov, O. V., Eggenschwiler, J. T., Hong, D. H., Anderson, K. V., and Li, T. (2004) Development 131, 2149–2159
10. Bai, X., Ma, D., Liu, A., Shen, X., Wang, Q. J., Liu, Y., and Jiang, Y. (2007) Science 318, 977–980
11. Barth, S., Nesper, J., Hasgall, P. A., Wirthner, R., Nytko, K. J., Edlich, F., Katschinski, D. M., Steidl, D. P., Wenger, R. H., and Camenisch, G. (2007)
