VCP/p97 AAA-ATPase Does Not Interact with the Endogenous Wild-Type Cystic Fibrosis Transmembrane Conductance Regulator

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel that is defective in cystic fibrosis. The most common mutation, $\Delta F508$ CFTR, is retained in the endoplasmic reticulum, retrotranslocated into the cytosol, and degraded by the proteasome. In a proteomics screen to identify $\Delta F508$ CFTR interacting proteins, we found that valosin-containing protein (VCP)/p97, a Type II AAA ATPase that is a component of the retrotranslocation machinery, binds $\Delta F508$ CFTR, and this interaction is stabilized by proteasomal inhibition. Since wild-type (WT) CFTR has been reported to be inefficiently processed during biogenesis with as much as 75% of the newly synthesized protein degraded by the proteasome, we examined the VCP interaction in Calu-3, T-84, and 16HBE, three epithelial cell lines that endogenously express WT CFTR. The results indicate that when WT CFTR processing is efficient, as demonstrated in Calu-3 cells, VCP does not interact. Interestingly, overexpression of recombinant WT CFTR in Calu-3 cells results in inefficient processing and VCP interaction, demonstrating that CFTR processing efficiency and the VCP interaction are tightly coupled. Furthermore, induction of ER stress and activation of the unfolded protein response result in inefficient processing of WT CFTR in Calu-3 cells and promote the WT CFTR-VCP interaction. The results support the hypothesis that components of the retrotranslocation machinery such as VCP do not interact with CFTR in epithelial cells that endogenously express WT CFTR, since under normal conditions the processing of the WT protein is efficient.

Keywords: CFTR; p97/VCP; epithelium; ubiquitination; biogenesis

Cystic fibrosis (CF) transmembrane conductance regulator (CFTR), the defective gene product in CF, is an ATP-binding cassette (ABC) transporter found at the apical surface of a number of epithelial cell types, where it functions as a cAMP-regulated chloride channel (1). It consists of a regulatory domain connecting two homologous halves, each half containing six transmembrane segments and a nucleotide-binding domain (2). Over 1,400 mutations have been identified in the CFTR gene, but by far the most common disease-causing mutation is $\Delta F508$, a deletion of phenylalanine at position 508 (CF Genetic Analysis Consortium; <http://www.genet.sickkids.on.ca/cftr>).

The $\Delta F508$ mutation causes CFTR to be retained in the endoplasmic reticulum (ER), retrotranslocated into the cytosol, and degraded through the ER-associated degradative pathway

CLINICAL RELEVANCE

Currently it is thought that, like mutant $\Delta F508$ cystic fibrosis transmembrane conductance regulator (CFTR), most wild-type (WT) CFTR is degraded during processing. In this study, WT CFTR is degraded only under endoplasmic reticulum stress conditions, providing stronger support for WT CFTR's efficient processing in native epithelium.

(ERAD) by the proteasome (3–6). Given that $\Delta F508$ CFTR retains some biological activity (9) and that more than 90% of patients with CF have at least one allele of $\Delta F508$ CFTR, there is considerable interest in understanding the molecular mechanisms controlling $\Delta F508$ CFTR biogenesis and degradation, particularly with regard to how $\Delta F508$ CFTR differs from the wild-type (WT) protein. Early studies indicate that not only the $\Delta F508$ mutant, but also a significant fraction of the newly synthesized WT CFTR is subjected to ERAD (4, 5). In contrast, recent studies suggest that under physiologic conditions, endogenous WT CFTR processing is efficient (7). Furthermore, efficient maturation of endogenous WT CFTR is compromised under ER stress conditions when the unfolded protein response (UPR) is activated (8).

In the present studies, a proteomics screen using two-dimensional gel analysis and MALDI-TOF mass spectroscopy was employed to identify specific CFTR co-precipitating proteins. Among the many proteins that were identified in this screen, three known interacting proteins were identified (Hsp70, Hsc70, and Hsp90), confirming the effectiveness of the screen. A fourth protein, valosin-containing protein (VCP)/p97, was also detected. This interaction was confirmed by reciprocal immunoprecipitations and Western blot analysis. In cell lines endogenously expressing WT CFTR, such as Calu-3, no VCP interaction could be detected. If recombinant WT CFTR was expressed in the Calu-3 cells, however, or if the cells were undergoing ER stress, then the VCP/WT CFTR interaction was detected. The results support the view that WT CFTR is efficiently processed in epithelial cell lines that endogenously express CFTR.

MATERIALS AND METHODS

Cells and Culture Conditions

HeLa, Calu-3, CFPAC-1, T84, and 16HBE cells were obtained from ATCC (Manassas, VA). HeLa, Calu-3, and 16HBE cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Calu-3 and 16HBE medium was also supplemented with 2 mM glutamine, 1mM sodium pyruvate, and 0.1 mM nonessential amino acids. CFPAC-1 cells were maintained in Iscove's modification of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. T84 cells were maintained in a 50/50 mix

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of DMEM and factor 12 media (DMEM/F12) supplemented with 10% FBS and 2 mM glutamine. HeLa cells stably expressing WT or Δ F508 CFTR under the cytomegalovirus (CMV) promoter (HeLa WT and HeLa Δ F), and Calu-3 cells stably expressing recombinant WT CFTR under the CMV promoter (Calu-3+WT) were stably transduced with a TranzVector (Tranzyme Corp., Birmingham, AL) as previously described (10–12) and maintained in selection medium (1–5 μ g/ml puromycin). Calu-3, Calu-3+WT, CFPAC-1, T84, and 16HBE cells used in the experiments were carefully maintained and only used at a low passage number (< 20).

Immunoprecipitations

For CFTR immunoprecipitation, cells were lysed and scraped in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris, pH 8.0). For co-immunoprecipitation, cells were lysed in 1% digitonin, 2.5 mM HEPES, 10.0 mM CaCl_2 , pH 7.6. All lysis buffers were supplemented with a protease inhibitor cocktail (Complete Mini; Roche, Indianapolis, IN). CFTR was immunoprecipitated using Protein A-immobilized agarose beads and antibodies to CFTR's C-terminus (24-1, # HB-11947; ATCC) or to the first nucleotide-binding domain. VCP was immunoprecipitated with Protein G-immobilized agarose beads and mouse monoclonal anti-VCP (Abcam, Cambridge, MA). For co-immunoprecipitation and two-dimensional gel analysis, antibodies were covalently coupled to agarose beads before use (ProFound Mammalian Co-IP Kit; Pierce, Rockford, IL).

Two-Dimensional Gel Analysis

CFTR was immunoprecipitated from HeLa cells expressing either WT or Δ F508 CFTR using the 24-1 antibody coupled to agarose beads. CFTR-interacting proteins were eluted from beads in ImmunoPure IgG Elution Buffer, pH 2.8 (Pierce) supplemented with 15 mM 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC; Avanti Polar Lipids, Alabaster, AL). Eluted protein samples were neutralized and diluted in rehydration buffer (7 M urea, 2 M thiourea, 15 mM DHPC, 1% NP-40, 5 mM tributylphosphate [TBP], and 0.5% ampholytes pI 3–10; Bio-Rad, Hercules, CA). To separate proteins by isoelectric point (pI), 200 μ l of the sample was used to rehydrate an 11-cm 3–10 linear gradient immobilized pH gradient (IPG) strip, which was then focused using an IPGphor II isoelectric focusing unit (Amersham, Piscataway, NJ) at 50 μ A/strip and 20°C. To separate proteins by size, the strips were then equilibrated by two washes in DTT buffer (6 M urea, 20% glycerol, 50 mM tris-HCl pH 8.8, 2% SDS, 65 mM DTT, and trace bromophenol blue) and one wash in iodoacetamide buffer (6 M urea, 20% glycerol, 50 mM tris-HCl pH 8.8, 2% SDS, 2.5% iodoacetamide, and trace bromophenol blue). Proteins on the strip were electrophoresed on Criterion two-dimensional gels (8–16%) at 200 V, fixed for 1 h in 40% methanol/10% acetic acid, and stained overnight with Sypro Ruby (Molecular Probes, Carlsbad, CA). After three 1-h destains, gels were imaged in ProXPRESS (PerkinElmer Life Sciences, Waltham, MA) at 100- μ m resolution.

Mass Spectrometry

Mass spectrometric analysis was completed by the UAB Proteomics Facility. Two-dimensional gel plug samples were destained completely by three 30-min washes in 50% 25 mM ammonium bicarbonate/50% acetonitrile, followed by a 10-min wash with 25 mM ammonium bicarbonate. Samples were digested with trypsin (Roche) for 16 h at 37°C. Resulting peptide fragments were extracted using a 1:1 solution of 5% formic acid and acetonitrile, dried in a Speed-Vac (Savant; GMI, Ramsey, MN), and resuspended in 0.1% formic acid. Samples were then desalted (C¹⁸ ZipTips; Millipore, Billerica, CA), diluted in a saturated solution of α -cyano-4-hydroxycinnamic acid (CHCA) matrix, applied to 96-well plates, dried, and analyzed by MALDI-TOF MS. The Voyager DE-Pro was used to collect spectra on the samples, and the MASCOT search database was used to identify the proteins (www.matrixscience.com).

In Vitro Phosphorylation and Western Blot

Immunoprecipitated CFTR was *in vitro* phosphorylated using cAMP-dependent protein kinase A (Promega, Madison, WI) and γ -³²P-labeled ATP (PerkinElmer Life Sciences), and analyzed as described previously (13). For Western blot analysis, CFTR was detected with polyclonal

rabbit antibody recognizing the second nucleotide-binding domain or 24-1; VCP was detected with monoclonal mouse anti-VCP (BD Transduction Laboratories, San Jose, CA); actin was detected with polyclonal rabbit anti-actin (Sigma, St. Louis, MO); polyubiquitinated protein conjugates were detected using an antibody recognizing polyubiquitinated conjugates, but not monoubiquitinated conjugates or free ubiquitin (FK-1; Biomol, Plymouth Meeting, PA). Proteins were visualized using horseradish peroxidase-labeled anti-mouse IgG or IgM, or anti-rabbit IgG (Bio-Rad) and a chemiluminescent substrate (SuperSignal West Pico HRP substrate; Bio-Rad).

Real-Time PCR Analyses

Total cellular RNA was isolated using Qiagen RNeasy Mini Kit (Cat. No. 74106; Qiagen, Valencia, CA). RNA samples were stored at –20°C. RT-PCR was performed using TaqMan One-Step RT-PCR Master Mix Reagents (Cat. No. 4309169; Applied Biosystems, Foster City, CA) containing Carboxy-X-rhodamine (ROX) as a passive reference to normalize non-PCR-related signal in each reaction. Probes were conjugated with carboxyfluorescein (FAM) at 5'-end and a non-fluorescent quencher at 3'-end. TaqMan assays use the 5' exonuclease activity of the DNA polymerase to free the fluorescent dye from the quencher during the PCR reaction. A proportional increase in dye concentration results in a crescent absolute fluorescence. Fluorescent probe sequence: CFTR5UTR-TS7M2 FAM. Nonfluorescent quencher: FAM-CTGCCGCTCAACCC. CFTR was amplified using CFTR5UTR-TS7F and CFTR5UTR-TS7R (GACATCACAGCAGGTCAGAGAAAA and GCTCCTAATGCCAAAGACCTACTAC, ID: CFTR5UTR-TS7). TaqMan RT-PCR reaction was performed in 25 μ l final volume containing 5 μ l (10 $\times$ dilution of stock) RNA sample, TaqMan Universal PCR Master Mix, No AmpErase UNG (2 $\times$) 12.5 μ l, MultiScribe Reverse Transcriptase and RNase Inhibitor (40 $\times$) 0.625 μ l, Primers and Probe (20 $\times$) 1.25 μ l, RNase-free water 5.625 μ l. Quantitative real-time PCR was performed using ABI PRISM 7500 Sequence Detection System (SDS). Five 1 log serial dilution reactions were performed in duplicates. Data were exported from ABI PRISM 7500 SDS software into Microsoft Excel and analyzed using the relative standard curve method. Ct values for each serial dilution were plotted in Microsoft Excel and the values were calculated from the y-intercept and slope of the standard curve using Excel Trendline option. These values were then used to calculate the input amount of mRNA samples. The input amount of target mRNA was normalized to GAPDH mRNA as an endogenous control.

Metabolic Pulse-Chase

Metabolic pulse-chase experiments were conducted as previously described (7). Briefly, cells were cysteine/methionine starved for 30 min, followed by a 30-min pulse labeling with 300 μ Ci/mL ³⁵S-methionine/cysteine (PerkinElmer Life Sciences). Cells were then lysed at indicated time points and CFTR was immunoprecipitated with 24-1 and analyzed by SDS-PAGE as described above. After visualization by autoradiography, maturation efficiency was quantified by comparing band B density at 0 time to that of labeled band C at 4 h using IPLab software, as previously described (14).

Induction of ER Stress

Cells were treated with 100 mM ALLN (Sigma) for the time intervals specified, and spliced XBP1 mRNA levels were measured using real-time RT-PCR (assay ID: Hs00231936_ml) as described previously (8).

RESULTS

Identification of VCP/p97 as a Δ F508 CFTR-Associated Protein

To compare binding partners between Δ F508 and WT CFTR, we examined a number of cell lines expressing either Δ F508 or WT CFTR, including an airway epithelial cell line that endogenously expresses WT CFTR, Calu-3. Our rationale was that we wanted to examine binding partners in a number of cell lines that express high levels of CFTR. The relative level of CFTR in each cell line is shown in Figure 1A.

Figure 1. Co-immunoprecipitation and two-dimensional gel analysis of $\Delta F508$ CFTR-interacting proteins. CFTR-associated proteins were co-immunoprecipitated from HeLa ΔF (10 mg protein), HeLa WT (18 mg protein), or Calu-3 (12 mg protein) cells using anti-CFTR antibody (24-1) and separated by two-dimensional gel electrophoresis (B, C, and E). Boxes represent sections of the gels detailed in Figure 2A. Parental HeLa cell lysates (without CFTR expression) immunoprecipitated with anti-CFTR antibody (D) and Calu-3 cell lysates immunoprecipitated with non-immune mouse IgG (F) are shown as controls. Sypro Ruby-stained gels were analyzed, and selected protein spots pulled down with $\Delta F508$ CFTR were identified by mass spectroscopy. Gels shown are representative of four to seven experiments. To demonstrate the relative amounts of CFTR in these cell lines, CFTR was immunoprecipitated from 500 μ g lysate using 24-1, labeled by *in vitro* phosphorylation using protein kinase A and γ - 32 P-labeled ATP, and detected by autoradiography (A).

To identify the binding partners, a proteomic screen based on co-immunoprecipitation, two-dimensional gel electrophoresis, and mass spectroscopic analysis was carried out. HeLa ΔF , HeLa WT, and Calu-3 cells were lysed in co-immunoprecipitation buffer (1% digitonin/2.5 mM HEPES, 10.0 mM CaCl_2 , pH 7.6), immunoprecipitated with anti-CFTR antibody, and CFTR-associated protein complexes were resolved by two-dimensional gel analysis. Calu-3 cells lysates immunoprecipitated with nonimmune IgG and HeLa cell lysates without CFTR expression (HeLa parental) immunoprecipitated with anti-CFTR antibody were tested as controls to assure selection of CFTR-specific binding partners (Figures 1B–1F). The results indicate that there were a large number of interacting proteins in the HeLa $\Delta F508$ and HeLa WT cells, but less co-immunoprecipitated proteins in the Calu-3 cells that endogenously express WT CFTR.

Spots of interest were excised and subjected to tryptic digestion and mass spectroscopic analysis. Spots were chosen for analysis if they were seen in all three cell lines, but not the controls, or if they differed between the endogenously expressing Calu-3 cells and the HeLa cell lines that heterologously express either WT or $\Delta F508$ CFTR. The protein matches identified by the MASCOT software that are relevant to this study are shown in Table 1. Three of the spots common to all three cell lines were identified as Hsp70 (15), Hsc70 (16), and Hsp90 (17) (Figure 2A), three known binding partners of CFTR. Each of these interactions was confirmed by co-immunoprecipitation and Western blot (Figure 2C, *insets*). During the mass spectroscopic analysis, a novel CFTR binding partner, VCP, was also identified (Figures 2A and 2B) from a spot present in the gels from both HeLa cell lines but absent in gels from Calu-3 cells. Sixteen VCP

TABLE 1. PROTEOMIC CHARACTERIZATION OF POLYPEPTIDES FROM A CFTR IMMUNOPRECIPITATION IN HELa CELLS

Name	gi Accession No.	Predicted PI	Observed PI	Predicted MW (kD)	Observed MW (kD)	Mowse Score	Number of Peptides Matched
Hsp 70	62089222	5.97	5.401	77.448	67.205	85	7
Hsc 70	57086907	5.37	5.398	70.854	65.372	71	5
Hsp 90 alpha 2	61656603	5.09	4.945	98.052	85.393	101	9
Hsp 90 beta 1	34304590	4.97	4.945	83.212	85.393	110	16
VCP/p97	6005942	5.214	5.14	97.14	89.266	118	16

Definition of abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; MW, molecular weight; PI, isoelectric point; VCP, valosin-containing protein.

Figure 2. Identification of $\Delta F508$ CFTR-interacting proteins by mass spectrometric analysis of proteins isolated from two-dimensional gels. Protein spots specified by the arrows in A were excised, destained, and trypsin digested for 16 h. The resulting peptide fragments were extracted from the gel, purified, and analyzed by MALDI-TOF mass spectroscopy. Peptide spectra were collected using Voyager DE-Pro. Using the MASCOT search database, Hsp70, Hsc70, and Hsp90 α and β , as well as VCP/p97, were identified as CFTR-binding partners (B–C). The interactions with Hsp70, Hsc70, and Hsp90 were confirmed by immunoprecipitating CFTR from HeLa ΔF cells and analyzing by SDS-PAGE and Western blot (C, inserts).

peptides were recognized by the mass spectrometry analysis of the tryptic digested gel spot, and the overall peptide match was statistically significant (Mowse score = 118; Table 1). Interestingly, VCP was not seen in the CFTR immunoprecipitation reactions from the airway epithelial cells (Figure 2A).

To confirm the VCP–CFTR interaction, co-immunoprecipitation experiments designed to pull down VCP with CFTR were performed in HeLa ΔF , HeLa WT, and Calu-3 cells. Parental HeLa cells lacking CFTR expression were used as negative controls. CFTR was immunoprecipitated from HeLa WT, HeLa ΔF , and Calu-3 cells after lysis in co-immunoprecipitation buffer. Co-immunoprecipitated proteins were separated by SDS-PAGE and Western blotted for VCP (Figure 3A). The results indicated that VCP associates with $\Delta F508$ CFTR, to a lesser extent with

WT CFTR from HeLa WT, and does not associate with WT CFTR from Calu-3 cells. In order to test whether the interaction depends on CFTR expression levels, relative amounts of CFTR in each of the cell lines were compared. Using equal amounts of cellular protein lysate, CFTR was immunoprecipitated, *in vitro* phosphorylated using cAMP-dependent protein kinase A and γ - ^{32}P -labeled ATP, and analyzed by SDS-PAGE and phosphorimaging. The results shown in Figure 3A (lower panel) indicate that although Calu-3 cells express the highest amount of CFTR, interaction between CFTR and VCP could not be demonstrated in these cells. Western blots performed on lysates containing equal amounts of protein (Figure 3A, middle panel) confirmed that the VCP levels were comparable in each of the cell lines tested. Taken together, these results indicate that there is

Figure 3. VCP is pulled down with CFTR expressed in HeLa cells but not in Calu-3 cells. (A) CFTR immunoprecipitation and VCP Western blot. Using lysates from HeLa parental, HeLa WT, HeLa ΔF, or Calu-3 cells, CFTR was immunoprecipitated using the 24-1 antibody covalently coupled to agarose beads and separated on 10% gels, followed by Western blotting and probing with monoclonal anti-VCP. Equal amounts of total cellular protein (1.5 mg) were immunoprecipitated from each cell line. VCP was pulled down with CFTR from HeLa WT and HeLa ΔF, but not from endogenous WT CFTR-expressing Calu-3 cell lysates (A, top panel). VCP expression levels were assessed by Western blotting. Ten micrograms of total protein from each cell type was separated on a 10% Tris-glycine gel, transferred to nitrocellulose membrane, and probed with monoclonal anti-VCP or polyclonal anti-actin (A, middle panels). CFTR expression levels were compared among cell types by immunoprecipitation of CFTR from 500 μg total protein using the 24-1 antibody and Protein A agarose beads, followed by *in vitro* phosphorylation (A, bottom panel). The gels shown above are representative of three experiments each. (B) Reverse co-immunoprecipitation of ΔF508 CFTR with VCP. VCP was immunoprecipitated from HeLa ΔF cell lysates using monoclonal anti-VCP and Protein G agarose beads (lane 3). HeLa ΔF cell lysates immunoprecipitated with nonimmune mouse IgG (lane 4) were used as a control. Proteins were eluted from the beads, and CFTR was recaptured with 24-1 antibody and protein A agarose beads followed by *in vitro* phosphorylation using protein kinase A and γ-³²P-labeled ATP, separated on 6% gels, and visualized by autoradiography. To test the efficiency of CFTR recapture, CFTR was immunoprecipitated with anti-CFTR NBD1 polyclonal antibody and *in vitro* phosphorylated (lane 1, 10% load), or eluted and recaptured with 24-1 antibody followed by *in vitro* phosphorylation (lane 2, 10% load). CFTR was only present in HeLa ΔF lysates immunoprecipitated with anti-VCP (lane 3, 100% load), but not in cell lysates immunoprecipitated with nonimmune mouse IgG (lane 4, 100% load). Proteins that were co-immunoprecipitated with anti-VCP antibody and *in vitro* phosphorylated were separated and visualized by autoradiography as a control (lane 5, 100% load). Lane 5 illustrates that a large number of proteins associate with VCP.

an interaction between VCP and recombinant WT CFTR in HeLa cells, but not between VCP and endogenous WT CFTR in the Calu-3 cells.

To further establish the VCP and ΔF508 CFTR interaction, reciprocal co-immunoprecipitations were performed to pull down ΔF508 CFTR with VCP. Since VCP is an abundant protein (up to 1% of the protein lysate [18]), and CFTR levels are extremely low compared with other proteins, these experiments were performed as re-immunoprecipitation reactions to enhance the CFTR detection by *in vitro* phosphorylation. In these experiments, VCP was immunoprecipitated from ΔF508 CFTR-expressing HeLa cells lysed in co-immunoprecipitation buffer. After re-solubilization of VCP-associated protein complexes, CFTR was re-immunoprecipitated using anti-CFTR antibody and detected by *in vitro* phosphorylation (Figure 3B, lane 3). Direct immunoprecipitation and *in vitro* phosphorylation of CFTR (lane 1) and re-immunoprecipitation of CFTR after the first immunoprecipitation (lane 2) were performed as controls. This was done to assure that re-immunoprecipitation of CFTR is efficient under the conditions used for VCP co-immunoprecipitation. Protein complexes co-immunoprecipitated with anti-VCP antibody were also *in vitro* phosphorylated and separated on the same gel. This control indicated that a large number of proteins associate with VCP as expected (Figure 3B, lane 5). These reciprocal co-immunoprecipitation experiments confirm the association of ΔF508 CFTR with VCP.

VCP Interacts with ΔF508, but Not WT CFTR, in Cells that Endogenously Express CFTR

Given that the results in HeLa cells transduced with WT CFTR were different than cells that endogenously express CFTR (i.e., Calu-3 cells), we next examined three additional epithelial cell lines that endogenously express ΔF508 and WT CFTR: CFPAC-1 (human pancreatic ΔF508 homozygous cell line), T-84 (human colonic epithelial cell line expressing WT CFTR), and 16HBE (human bronchial epithelial cell line expressing WT CFTR) cells. Immunoprecipitation with anti-CFTR antibody and Western blot for VCP indicated that VCP associated with ΔF508 CFTR from HeLa cells and from CFPAC-1 cells, but it did not co-immunoprecipitate with WT CFTR in Calu-3, T-84, or 16HBE cells (Figure 4A). The relative level of CFTR protein and messenger RNA in these cell lines is shown in Figures 4B. To confirm that the relative amounts of VCP in the endogenous CFTR expressers were similar, Western blots performed on lysates containing equal amounts of protein (Figure 4C, upper panel). As an additional control, β-actin was also detected in the lysates using Western blot (Figure 4C, lower panel). These results show that even though the amount of ΔF508 CFTR protein is extremely low in the CFPAC-1 cells, an interaction with VCP could still be demonstrated. Furthermore, the WT protein does not interact with VCP in two other epithelial cell lines tested, T-84 and 16HBE (Figure 4A), indicating that WT CFTR does not interact with VCP in three cell lines that endogenously express WT CFTR.

The CFTR-VCP Interaction Requires Inefficient CFTR Processing

Given VCP's role in retrotranslocation of misfolded proteins and ERAD (19, 20), we tested the hypothesis that the WT protein does not interact with VCP in Calu-3 cells because WT CFTR is efficiently processed to the maturely glycosylated protein in these cells, with a corollary being that anything that disrupts CFTR processing in Calu-3 cells should promote its association with VCP. To test this hypothesis, Calu-3 cells expressing both endogenous WT and recombinant WT CFTR were

Figure 4. The VCP-ΔF508 CFTR interaction is independent of CFTR expression levels and is present in CFPAC-1 cells endogenously expressing ΔF508 CFTR. (A and B) VCP interacts with heterologous and endogenous ΔF508 CFTR. Using lysates from HeLa parental (5 mg), HeLa ΔF (5 mg), CFPAC-1 (17 mg), Calu-3 (4 mg), T84 (9 mg), or 16HBE (19 mg) cells, CFTR was immunoprecipitated using the 24-1 antibody covalently coupled to agarose beads. Immunoprecipitated proteins were separated on a 10% gel, transferred to nitrocellulose membrane, and probed with monoclonal anti-VCP (A). The gel shown is representative of three independent experiments. Amounts of material used for co-immunoprecipitation in A correspond to equivalent CFTR levels as demonstrated in B, in which CFTR was immunoprecipitated using 24-1, labeled by *in vitro* phosphorylation, and detected by autoradiography (B, upper panel). CFTR transcript levels were measured using TaqMan quantitative real-time PCR using GAPDH as a control. Samples were plotted as CFTR mRNA levels relative to GAPDH. Mean and SD of $n = 3$ samples amplified under the same conditions are shown (B, lower panel). VCP was pulled down with both recombinant (HeLa) and endogenous (CFPAC-1) ΔF508 CFTR, but no VCP was pulled down from T84 cells endogenously expressing WT CFTR. (C) Relative VCP and actin expression levels. The amount of VCP (upper panel) and actin (lower panel) in all endogenous CFTR-expressing cell lines was equivalent. Despite ΔF508 CFTR expression levels that were $> 1,000$ -fold lower in CFPAC-1 cells than the recombinant ΔF508 CFTR levels in HeLa ΔF cells, VCP was pulled down with both recombinant and endogenous ΔF508 CFTR.

tested (Calu-3+WT) (8). In contrast to the significantly higher CFTR mRNA levels in Calu-3+WT cells, steady-state CFTR expression levels of the parental Calu-3 and Calu-3+WT cells are similar (Figure 5A). To test if the Calu-3+WT cells had

altered processing efficiency compared to the parental Calu-3 cells, metabolic pulse-chase experiments were performed. In these experiments, we compared the amount of newly synthesized Band B CFTR (core-glycosylated form) and monitored its conversion to the fully glycosylated Band C CFTR in Calu-3 and Calu-3+WT cells. The results show that the conversion of the core-glycosylated CFTR to the maturely glycosylated form was efficient ($91 \pm 4\%$ [$n = 3$]) in the parental Calu-3 cells. In contrast, the maturation efficiency of WT CFTR in the Calu-3+WT cells was $55 \pm 8\%$ ($n = 3$), demonstrating that expression of the recombinant protein dramatically decreased the processing efficiency of WT CFTR (Figure 5B).

Given the processing efficiency differences in the two Calu-3 cell lines, we next tested if VCP interacted with WT CFTR in the Calu-3+WT cells. CFTR was immunoprecipitated from Calu-3 and Calu-3+WT cells and analyzed by Western blot for VCP. CFTR immunoprecipitated from CFPAC-1 cells was included as a positive control for VCP interaction. The results shown in Figure 6A indicate that VCP co-immunoprecipitates with WT CFTR in Calu-3+WT cells, but not in the parental Calu-3 cells. This supports the view that VCP interacts with WT CFTR only when the processing is compromised, and because Calu-3 cells normally process WT CFTR very efficiently, retrotranslocation and ERAD of endogenous CFTR does not occur under normal physiologic conditions.

Recent studies showed decreased CFTR maturation efficiency in Calu-3 cells after induction of ER stress and activation of the UPR (8). Based on this observation, we tested the hypothesis that when the UPR is activated and a larger fraction of endogenous WT CFTR becomes a substrate for ERAD, association with VCP becomes apparent. To test this, ER stress was induced using proteasome inhibition as described previously (8) and VCP/CFTR co-immunoprecipitations were performed. After 2 h or more of proteasome inhibition, VCP/CFTR association was apparent (Figure 6B). To ensure that VCP-CFTR interaction is related to the activation of the UPR, sXBP-1 mRNA levels were measured (Figure 6C). We treated parental Calu-3 cells with ALLN for various periods of time to induce the UPR. At each time point, we tested for an increase in sXBP-1 mRNA. A significant increase in sXBP mRNA ($2\times$) coincided with detectable VCP/CFTR interaction in these cells, suggesting that VCP-CFTR interaction is promoted by the UPR. Taken together, these results support the hypothesis that the interaction of VCP with CFTR is specific to the ERAD pathway, and that there is no interaction between VCP and WT CFTR under normal conditions because in Calu-3 cells CFTR is not a substrate of the proteasome. However, under conditions that lower WT CFTR maturation efficiency, such as overexpression of recombinant CFTR or activation of the UPR, an interaction between CFTR and VCP can be detected.

VCP Interacts with Polyubiquitinated ΔF508 CFTR

Since ΔF508 CFTR interacts with VCP, we next sought to determine if VCP interacted with the unmodified or ubiquitinated form of ΔF508 CFTR. In these experiments, VCP was immunoprecipitated, and CFTR was re-immunoprecipitated from the solubilized fraction, followed by SDS-PAGE and Western blot analysis for CFTR and polyubiquitin conjugates (Figure 7, left panels). As a control, CFTR was immunoprecipitated and Western blotted for CFTR and polyubiquitin from 10% of cell lysates (Figure 7, right panels). The results indicate that VCP-associated CFTR is polyubiquitinated. Interestingly, the molecular weight of the polyubiquitinated CFTR ladder that was pulled down with VCP was mostly below 250 kD, suggesting that the VCP association occurs early during CFTR retrotranslocation and ubiquitin modification process. In WT-expressing cells, namely

Figure 5. Expression of recombinant CFTR in Calu-3 cells decreases CFTR processing efficiency. Steady-state CFTR levels were compared in parental Calu-3 and Calu-3+WT cells by immunoprecipitating CFTR from 5 mg lysate using 24-1 and Protein A agarose beads. Immunoprecipitated proteins were separated on a 6% gel, transferred to nitrocellulose membrane, and probed with polyclonal anti-CFTR NBD-2 (A). To compare maturation efficiency in parental Calu-3 and Calu-3+WT, cells were pulse labeled with 35 S-methionine/cysteine, lysed at indicated time points, and CFTR was immunoprecipitated with 24-1 and analyzed by SDS-PAGE. After visualization by autoradiography, maturation efficiency was quantified by comparing labeled band B density to that of labeled band C using IPLab software as previously described (14).

HeLa WT and parental Calu-3 cells, total ubiquitinated CFTR levels were measured by immunoprecipitation for CFTR followed by Western blot for ubiquitin. In HeLa WT cells, we observed a low level of ubiquitination, but in Calu-3 cells, no ubiquitinated CFTR was detectable (data not shown). Together, these results support the idea that endogenous WT CFTR is not a substrate for ERAD, and that Δ F508 CFTR interacts with VCP en route to the proteasome.

DISCUSSION

The present study was designed to select Δ F508 CFTR binding partners. VCP, a component of the ER-to-proteasome retrotranslocation machinery in eukaryotic cells, was selected in co-immunoprecipitation experiments and identified using mass spectroscopy as Δ F508 CFTR-specific binding partner. The VCP-CFTR interaction was confirmed in co-immunoprecipitation experiments in endogenous and recombinant WT or Δ F508 CFTR-expressing cell lines. VCP is a Type II AAA ATPase that contains two conserved ATPase domains and functions as a chaperone. In addition to other various cellular activities, VCP is a part of the retrotranslocation machinery required for transport of misfolded proteins out of the ER before their degradation by the proteasome (20–23). VCP (Cdc48p in yeast) is part of a multiprotein complex containing other cofactors, including Ufd1p and Npl4p (23, 24). VCP and Ufd1 associate with unmodified proteins, an interaction that persists during the early stages of the polyubiquitination process.

Our results indicate that VCP interacts with the fraction of CFTR selected for ERAD early in the ubiquitin modification process. The predominant CFTR fractions interacting with VCP represent unmodified and lower molecular weight ubiquitin-modified fractions, suggesting that the interaction occurs in the ER membrane before ubiquitin modification and persists during the early stages of ubiquitination. This finding is consistent with a model proposed by Ye and colleagues in which binding of misfolded proteins by VCP precedes ubiquitination, and polyubiquitination occurs after the initial VCP interaction (23).

Our results showing that VCP interacts with Δ F508 CFTR are consistent with previous studies that reduce VCP activity, either using dominant-negative forms of VCP (25, 26) or using RNAi targeting VCP (27). In these studies, reduction of VCP extended the half-life of Δ F508 CFTR, led to its accumulation in the ER, or even promoted its rescue from ERAD, indicating that VCP function is important for Δ F508 ERAD. Recent studies show that while VCP is not absolutely required for CFTR degradation, it does greatly enhance the degradation of CFTR by aiding in the unfolding and retrotranslocation of the transmembrane regions (28). The ATPase activity of VCP is required for this retrotranslocation (23).

Our observation that VCP only interacts with WT CFTR when it is expressed as a recombinant protein is consistent with the model that WT CFTR biogenesis is inefficient in overexpression systems, and some of the newly synthesized WT CFTR is a substrate of the proteasome (7). This is in contrast to the results in Calu-3, T84, and 16HBE cells, in which no interaction could be demonstrated under physiologic conditions. Although a recent study demonstrates VCP immunoprecipitating with CFTR expressed in Calu-3 cells (29), we have repeatedly seen no such interaction under our stringent culture conditions, except when ER stress was induced in these cells (8). In contrast to the parental Calu-3 cell line, recombinant CFTR expression in Calu-3 cells results not only in inefficient maturation, but also in an interaction with VCP. Our finding that activation of the UPR by proteasomal inhibition promoted the interaction between CFTR and VCP in Calu-3 cells supports the previous finding that under these conditions, the maturation efficiency of CFTR is decreased (8). However, it is possible that a small amount of endogenous WT CFTR may be degraded by the proteasome even under physiologic conditions. Interestingly, we could not detect the association of WT CFTR with VCP, in spite of the fact that the Δ F508 CFTR association was apparent even in CFPAC-1 cells that express minimal levels of the mutant. Based on these results, it is clear that under physiologic conditions, the fraction of endogenous WT CFTR that is destined for

Figure 6. Expression of recombinant WT CFTR in Calu-3 cells promotes VCP-CFTR interaction. (A) VCP interacts with overexpressed, but not endogenous, WT CFTR in Calu-3 cells. WT or $\Delta F508$ CFTR was co-immunoprecipitated from Calu-3 parental (5 mg), Calu-3+WT (5 mg), or CFPAC-1 (19 mg) cells using the 24-1 antibody covalently coupled to agarose beads. Immunoprecipitated CFTR was separated on a 10% Tris-glycine gel, transferred to nitrocellulose membrane, and probed with monoclonal anti-VCP. VCP does not interact with endogenous CFTR in Calu-3 parental cells, but VCP was pulled down with WT CFTR from Calu-3+WT cells and with endogenous $\Delta F508$ CFTR from CFPAC-1 cells. (B) Time course for the ER stress-induced VCP-CFTR interaction. Parental Calu-3 cells were treated with 100 μ M ALLN for the indicated times before lysis and processing as above. The VCP-CFTR interaction appears after 2 h of ALLN treatment. (C) sXBP-1 mRNA levels in ALLN-treated Calu-3 cells over time. As a readout of ER stress induction, sXBP levels were measured using real-time PCR analysis of the same cell lysates at the time points indicated. All gels shown are representative of two independent experiments.

the proteasome must be lower than the amount of endogenous $\Delta F508$ CFTR synthesized in CFPAC-1 cells.

Our observation that the CFTR-VCP interaction occurs concomitantly with an increase in sXBP-1 is supported by the previous finding that endogenous CFTR maturation is compromised when the UPR is activated (8). In the three different epithelial cell lines endogenously expressing CFTR tested in this study, no interaction could be detected between endogenous CFTR and VCP unless the UPR was activated. Our finding that overexpression of recombinant WT CFTR in Calu-3 cells decreases maturation efficiency and concomitantly results in VCP/CFTR binding suggests that recombinant CFTR expression is regulated differently from endogenous CFTR, even in an epithelial cell line. Supporting evidence for this idea is found in a recent study in which WT CFTR was overexpressed in the bronchial epithelial cell line IB3-1. Inhibition of VCP using RNAi increased processing of WT CFTR to the mature form and promoted stabiliza-

Figure 7. VCP interacts with $\Delta F508$ CFTR early in the polyubiquitination process. HeLa ΔF cells were lysed in co-immunoprecipitation buffer and VCP was immunoprecipitated using mouse monoclonal anti-VCP antibody and Protein G agarose beads. After elution of immunoprecipitated proteins, CFTR was recaptured using 24-1 antibody and protein A agarose beads. Immunoprecipitated CFTR was then separated by SDS PAGE, Western blotted, and probed for CFTR (left panel, lane 1) or for polyubiquitin conjugates (left panel, lane 2). As control, CFTR was immunoprecipitated using the 24-1 antibody and Protein A agarose beads, separated by SDS PAGE, transferred to nitrocellulose membrane, and probed with polyclonal antibodies recognizing either NBD-2 of CFTR (right panel, lane 1) or polyubiquitin conjugates (right panel, lane 2). The majority of the polyubiquitinated $\Delta F508$ CFTR in HeLa ΔF cells appears as a broad band. The population of ubiquitinated $\Delta F508$ CFTR that was pulled down with VCP appears as a ladder of bands between 150 and 250 kD. A large fraction of $\Delta F508$ CFTR pulled down with VCP runs as a sharp band just above 150 kD, suggesting that VCP interacts with $\Delta F508$ CFTR at a distinct, early point in the ubiquitination process. The gel shown is representative of four independent experiments.

tion of band C on the cell surface (27), suggesting that overexpressed WT CFTR is a substrate for ERAD, and therefore it associates with VCP, even in a bronchial cell line. In another recent study conducted in the Calu-3+WT cell line, induction of ER stress by proteasome inhibition decreased endogenous CFTR mRNA levels, but had no effect on the heterologous CFTR mRNA (8). This finding suggests that endogenous CFTR mRNA levels are tightly regulated, and this regulation is absent in recombinant CFTR.

It is also important to point out that all cell lines tested in this study contained equivalent VCP levels, but widely varying CFTR levels. Although a critical component for the VCP-CFTR interaction appears to be inefficient processing, the relative expression levels of CFTR and VCP are not important. CFPAC-1 cells, for instance, have the lowest levels of CFTR of any cell line tested for this study, but since the $\Delta F508$ CFTR they express is completely degraded by ERAD, a VCP interaction is easily detected. In contrast, Calu-3, T84, and 16HBE cells have much higher mRNA levels of endogenous WT CFTR ($\sim 400\times$, $100\times$, and $75\times$, respectively), but no VCP-CFTR interaction is detected in these cells under normal conditions. These results support the hypothesis that VCP interacts specifically with CFTR destined for ERAD.

Based on these studies, we propose that analyzing the interaction between VCP and CFTR in other cell lines could be used to monitor whether CFTR is destined for ERAD. Furthermore, this interaction can be used to monitor biogenesis efficiency in cell lines in which metabolic pulse-chase analysis is not possible, such as primary epithelia.

In summary, a proteomic screen for binding partners in $\Delta F508$ CFTR-expressing cells identified VCP, a component of the ubiquitin-proteasomal pathway in ERAD. Our inability to detect this interaction in cell lines that endogenously express WT CFTR confirms and supports the idea that WT CFTR biogenesis is efficient in these cell lines and that WT CFTR is normally not a substrate of the proteasome. This type of analysis also suggests that a similar search for WT and $\Delta F508$ CFTR binding partners in airway epithelia would be useful in identifying those interactions that have been lost by $\Delta F508$ CFTR, not only during its biogenesis, but also after rescue at the cell surface. Identification of the normal binding partners of WT CFTR during its biogenesis and after its delivery to the cell surface is critical for understanding the defects associated with $\Delta F508$ CFTR in the secretory pathway and at the cell surface.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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