Non-conventional Trafficking of the Cystic Fibrosis Transmembrane Conductance Regulator through the Early Secretory Pathway

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The mechanism(s) of cystic fibrosis transmembrane conductance regulator (CFTR) trafficking from the endoplasmic reticulum (ER) through the Golgi apparatus, the step impaired in individuals afflicted with the prevalent CFTR-ΔF508 mutation leading to cystic fibrosis, is largely unknown. Recent morphological observations suggested that CFTR is largely absent from the Golgi in situ (Bannykh, S. I., Bannykh, G. I., Fish, K. N., Moyer, B. D., Riordan, J. R., and Balch, W. E. (2000) Traffic 1, 852–870), raising the possibility of a novel trafficking pathway through the early secretory pathway. We now report that export of CFTR from the ER is regulated by the conventional coat protein complex II (COPII) in all cell types tested. Remarkably, in a cell type-specific manner, processing of CFTR from the core-glycosylated (band B) ER form to the complex-glycosylated (band C) isoform followed a non-conventional pathway that was insensitive to dominant negative Arf1, Rab1a/Rab2 GTPases, or the SNAP REceptor (SNARE) component syntaxin 5, all of which block the conventional trafficking pathway from the ER to the Golgi. Moreover, CFTR transport through the non-conventional pathway was potently blocked by overexpression of the late endosomal target-SNARE syntaxin 13, suggesting that recycling through a late Golgi/endosomal system was a prerequisite for CFTR maturation. We conclude that CFTR transport in the early secretory pathway can involve a novel pathway between the ER and late Golgi/endosomal compartments that may influence developmental expression of CFTR on the cell surface in polarized epithelial cells.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-regulated chloride channel polarized to the

apical membrane in numerous epithelia including those found in lung, pancreas, intestine, and kidney (1, 2). Although nearly 1000 mutations have been identified in the CFTR gene, ~70% of CF chromosomes contain the ΔF508 mutation, which leads to severe forms of the genetic disease cystic fibrosis (CF) (3–5). Deletion of F508 prevents proper folding and trafficking of CFTR from the endoplasmic reticulum (ER) to the plasma membrane (6, 7). Both wild-type CFTR and CFTR-ΔF508 can be detected in ER and ER-Golgi transport intermediates, but only wild-type CFTR is readily expressed at the cell surface (2, 8, 9). Both newly synthesized wild-type CFTR and CFTR-ΔF508 molecules that neither fold productively nor achieve an ER export-competent conformation are eliminated by covalent addition of ubiquitin followed by degradation via the proteosome (9, 11). Whereas ~20–40% of wild-type CFTR nascent chains become properly folded, are exported from the ER, and acquire complex carbohydrates characteristic of passage through the Golgi apparatus, negligible levels of CFTR-ΔF508 follow this pathway, and CFTR-ΔF508 is quantitatively degraded (12, 13).

To rationally develop therapeutic means to stimulate CFTR-ΔF508 trafficking from the ER to the cell surface, it is first necessary to understand the mechanisms and pathways directing wild-type CFTR trafficking. By using morphological analyses, we recently reported that although wild-type CFTR was readily detectable in ER membranes, ER-Golgi transport intermediates, endosomes, and the plasma membrane, it was largely absent from the Golgi stack in vivo and in situ (14). However, the molecular mechanism of CFTR export from the ER as well as the biochemical foundation for its absence from Golgi cisternae were not addressed. We have therefore examined the biosynthetic pathway of CFTR trafficking through the early secretory pathway at the molecular level. We report that not only is the trafficking pathway by which CFTR acquires complex carbohydrates cell type-specific but that CFTR maturation can utilize a non-conventional pathway whereby the protein is first likely to be transported to distal Golgi compartments and/or the endosomal system prior to retrieval to earlier Golgi compartments for oligosaccharide processing to the complex form. Pharmacological modulation of this non-conventional pathway may be useful to selectively stimulate CFTR-ΔF508 transport to the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies were used in this study: a monoclonal antibody (M5A7) against the second nucleotide-binding domain of CFTR (15), a monoclonal antibody (p5D4) against the C-terminal cytoplasmic tail of the vesicular stomatitis virus G protein (VSV-G) (16), and a polyclonal antibody against Rab6 (B. Goud, Institut Curie, Paris, France) (17). Protein G-Sepharose 4 Fast Flow beads were from Amersham Biosciences; BFA was from Calbiochem; Complete Protease In-

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hibitor Cocktail Tablets were from Roche Molecular Biochemicals; Li- 
poetAMINE Plus Reagent, Opti-MEM I reduced serum medium, 
DMEM, and α-minimum Eagle’s medium were from Invitrogen; and 
Easy Tag Express Protein Labeling 35S-Met Mix was from PerkinElmer 
Life Sciences.

Vaccinia Virus Infection and Transfection—BHK, HeLa, and HEK- 
293T cells were maintained in DMEM supplemented with 10% fetal 
bovine serum and 100 units/ml each of penicillin and streptomycin in 
a 5% humidified CO2 incubator at 37 °C. CHO and CHO-15B cells were 
maintained α-minimum Eagle’s medium as above. Infection with T7 
RNA polymerase-recombinant vaccinia virus and transfection were per- 
formed as described (18) with minor modifications. Briefly, BHK cells 
(1.5 × 105) were plated in 60-mm cell culture dishes 1 day prior to 
experimentation. Before infection, cells were washed twice with Opti- 
MEM I and then infected with recombinant vaccinia virus expressing 
the T7 RNA polymerase (19, 20) at a multiplicity of 10 plaque-forming 
units/cell in 1 ml of Opti-MEM I for 30 min with rocking in a 5% 
humidified CO2 incubator at 37 °C. Infection media were removed; cells 
were washed twice with Opti-MEM I, and cells were co-transfected with 
7 μg of pcDNA3.1 wild-type CFTR and 7 μg of pcDNA3.1/pET vector 
(21) containing trafficking machinery using LipofectAMINE Plus Rea-
gent, following the manufacturer’s instructions. Transfection medium 
was aspirated after 3 h and replaced with DMEM containing 10% fetal 
bovine serum for an additional 5–30 min with 500 U of TranSMIS-l and then 
chased in DMEM containing 10% fetal calf serum and 10 mM cold 
methionine. For experiments utilizing BFA, cells were chased in the 
above medium containing 5 μM cold methionine. For experiments utilizing 
specific antibodies (data not shown) to ensure typical expression 
levels of 5–10-fold over endogenous protein levels (21). Experiments 
comparing effects of trafficking machinery components on transport of 
VSV-G and CFTR were performed in parallel.

Transport Analysis—Transport analysis of VSV-G was performed as 
described previously (18). For transport analysis of CFTR, medium was 
removed after 8–10 h of transfection, and cells were washed twice 
with phosphate-buffered saline (pre-warmed to 37 °C) and incubated for 
30 min in methionine-deficient minimum Eagle’s medium. Cells were 
radialabeled for 20–30 min with 500 μCi of Trans35S-l and then 
chased in DMEM containing 10% fetal calf serum and 10 mM cold 
methionine. For experiments utilizing BFA, cells were chased in the 
above medium containing 5 μM cold methionine and washed twice 
in ethanol as control. After the indicated chase periods, cells were 
treated with enhancing solution (125 mM sodium salicylate, 35% meth-
ol) for 1 h and dried. Signals were developed by autoradiography and 
scanned by a computer software (Sendry, Pharmacia, Piscataway, NJ).

We first examined the role of the COPII complex, composed of 
the small molecular weight GTPase Sar1 and the hetero- 
erodimeric Sec23–24 and Sec13–31 complexes in CFTR export 
from the ER (26–29). To block COPII vesicle function in BHK 
cells, we co-expressed wild-type CFTR with Sar1-GTP(H79G), 
a constitutively active mutant locked in the GTP-bound form 
that inhibits vesicle uncoating leading to the accumulation of 
ER to Golgi transport intermediates (28, 30). Co-expression of 
Sar1 or other components of the vesicular trafficking pathway 
with CFTR (see below) generally leads to a 5–10-fold elevated 
level of the component over the endogenous pool, consistent 
with previous results (18, 23–26) (data not shown). Following 
labeling of cells with a pulse of [35S]methionine, delivery of 
CFTR to the Golgi was measured by the conversion of the 
~140–150-kDa core-glycosylated band B pre-Golgi form to 
~170–190-kDa complex glycosylated band C isofrom diagnos-
tic of processing by Golgi-associated mannosidases and glyco-
sytransferases leading to the addition of polylactosamine (31) 
(Fig. 1, A–C).

In control cells, ~30% of CFTR was processed to the mature 
band C glycoisoform over 3 h, consistent with the efficiency and 
rate of CFTR maturation reported in other stably transfected 
cell lines (12, 13). By contrast, no detectable levels of CFTR in 
the band C glycoisoform were observed following overexpression 
of Sar1-GTP(H79G) (Fig. 1B). As a control, under identical 
conditions we also tested the effect of Sar1-GTP(H79G) on the 
processing of the reporter cargo type 1 transmembrane protein 
vesicular stomatitis virus glycoprotein (VSV-G) from the end-
doglycosidase H (endo H)-sensitive pre-Golgi form to the endo 
H-resistant, Golgi-processed glycoisoform as described previ-
ously (28, 30). Consistent with previous findings (26, 30, 32–34), 
transport of VSV-G was potently inhibited by the Sar1-
GTP(H79G) mutant (Fig. 1D). Thus, export of CFTR from the 
ER, like VSV-G, is dependent on a COPII-mediated mecha-
nism. Consistent with this interpretation, morphological analy-
ses of cells expressing wild-type CFTR using high resolution 
deconvolution microscopy showed a significant co-localization 
of wild-type CFTR with the Sar1 GTPase (~70% of Sar1 con-
taining punctate structures) and the Sec23 component of the 
COPII coat complex (~40% of punctate Sec23 containing 
structures) (Fig. 1E) that is significantly reduced in cells express-
ing the ΔF508 mutant that cannot exit the ER (~15% of Sar1 
containing punctate structures; <10% of Sec23 containing 
structures) (Fig. 1E) (35).

To quantitatively express the efficiency of maturation of 
CFTR from band B to band C glycoisofoms, we compared the 
band B/C ratio of CFTR band C to CFTR band B as a function of time. If 
the band C/B ratio remains unaltered relative to the 
control by co-expression with dominant negative mutants, 
CFTR processing to the mature glycoisoform is not affected. 
If the C/B ratio is reduced, CFTR processing to the mature glyco-
isoform is inhibited. By using this method, we found that 
although expression of CFTR alone exhibited a C/B ratio of ~6 
after a 3-h chase period, co-transfection with Sar1-GTP(H79G) 
yielded a C/B ratio of ~0.1 (Fig. 1C). Interestingly, not only was 
CFTR processing blocked by the Sar1-GTP(H79G) mutant but 
Sar1-GTP(H79G) stabilized immature CFTR band B and re-
duced its rate of degradation ~3–6-fold (Fig. 1A).

CFTR Maturation Is Not Blocked by Dominant Negative 
Arf1-GTP—Whereas the above results are consistent with the 
generally accepted view that the COPII machinery is involved in 
the export of cargo proteins from the ER (26, 29, 32–34, 36), 
the step following export from the ER generally requires the function(s) of the coat complex I (COPI) machinery that is 
recruited to pre-Golgi intermediates consisting of vesicular tu-
cular clusters (VTCs) (14) that are potentially formed from the 
fusion of COPII vesicles (37). COPI components include the 
small molecular weight GTPase Arf1 and the coatomer coat

RESULTS

CFTR Maturation Is Blocked by Dominant Negative Sar1-
GTP—To investigate the mechanism(s) of wild-type CFTR traf-
ficking in the early secretory pathway, we used a recombinant 
T7-vaccinia virus expression system (19, 20) to transiently 
co-express wild-type CFTR and key components regulating the 
formation, targeting, and fusion of endoplasmic reticulum 
(ER)-derived transport intermediates with Golgi membranes. 
This approach has been used previously (23) to examine trans-
port of CFTR through the secretory pathway, the functional 
role of the coat (Arf1, coatomer II (COPII)), and coatomer complex I (COPI) involved in transport vesicle assembly, and 
the role of Rab GTPases and SNARE Receptor (SNARE) proteins 
included in membrane fusion in the trafficking of cargo through 
the early secretory pathway (18, 23–26).

We first examined the role of the COPII complex, composed of 
the small molecular weight GTPase Sar1 and the het-
The function of the COPI coat complex remains controversial. COPI may function to regulate anterograde transport of VTCs from the ER to the Golgi stack, transport within the Golgi stack, and/or the retrograde transport of recycling components from pre-Golgi and Golgi membranes to the ER (27, 38, 39).

To investigate the requirement for COPI in the processing of CFTR, we co-expressed CFTR with Arf1-GTP(Q71L), a dominant negative mutant locked in the GTP-bound form that inhibits COPI function.

**FIG. 1.** CFTR processing is blocked by dominant negative Sar1-GTP. A–C, BHK cells were co-transfected with pcDNA3.1-CFTR and empty vector (mock) or with pcDNA3.1-CFTR and pcDNA3.1-GST-Sar1-GTP(H79G), and CFTR processing was monitored as described under “Experimental Procedures.” Expression levels of transport components were monitored by immunoblotting with specific antibodies as described under “Experimental Procedures.” A, quantitation of CFTR band B (% of total B plus C at the 0 time point). Inset, representative autoradiogram is shown. B, quantitation of the CFTR band C glycoisoform (% of total B plus C at the 0 time point). C, ratio of CFTR band C to band B plotted as a function of time. D, inhibitory effect of Sar1-GTP(H79G) on processing of VSV-G from the endo H-sensitive (endo H⁺) ER form to the endo H-resistant (endo H⁻) Golgi form.

**FIG. 2.** CFTR processing is independent of Arf1 function in BHK cells. A–C, BHK cells were co-transfected with pcDNA3.1-CFTR and empty vector or with pcDNA3.1-CFTR and pET-Arf1-GTP(Q71L). A, quantitation of CFTR band B. Inset, representative autoradiogram is shown. B, quantitation of the CFTR band C glycoisoform. C, ratio of CFTR band C to band B plotted as a function of time. D, inhibitory effect of Arf1-GTP(Q71L) on processing of VSV-G from the endo H-sensitive (endo H⁺) ER form to the endo H-resistant (endo H⁻) Golgi form.

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**FIG. 3.** CFTR processing is independent of Arf1 function in BHK cells. A–C, BHK cells were co-transfected with pcDNA3.1-CFTR and empty vector or with pcDNA3.1-CFTR and pET-Arf1-GTP(Q71L). A, quantitation of CFTR band B. Inset, representative autoradiogram is shown. B, quantitation of the CFTR band C glycoisoform. C, ratio of CFTR band C to band B plotted as a function of time. D, inhibitory effect of Arf1-GTP(Q71L) on processing of VSV-G from the endo H-sensitive (endo H⁺) ER form to the endo H-resistant (endo H⁻) Golgi form.

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hobits ER to Golgi transport of VSV-G by blocking COPI coat disassembly (24). Control experiments demonstrated that the Arf1-GTP(Q71L) mutant was functional under these conditions as it stimulated coatamer (β-COP) recruitment to Golgi membranes (data not shown) and completely blocked the processing of VSV-G to the endo H-resistant form (Fig. 2D) as reported previously (24, 40). Remarkably, overexpression of Arf1-GTP(Q71L) in BHK cells had no effect on the rate of CFTR band B degradation, the rate of CFTR band C formation, or the CFTR C/B ratio (Fig. 2, A–C). Thus, CFTR maturation is Arf1-independent, suggesting that neither the potential role of Arf1 in anterograde transport nor retrograde recycling of transport components perturbs egress or degradation of wild-type CFTR from the ER in BHK cells.

**CFTR Maturation Is Not Blocked by Dominant Negative Rab1a or Rab2**—Rab1 and Rab2 are members of the ubiquitous Rab family of small molecular weight GTPases that are required for vesicle targeting and fusion in both the exocytic and endocytic pathways (41, 42). Rab1 regulates both anterograde trafficking from the ER to the cis-Golgi network and intra-Golgi transport, whereas Rab2 has been proposed to control retrograde transport from post-ER, pre-Golgi intermediates to the ER (18, 43–46).

To examine the requirement for Rab1 and Rab2 function in CFTR maturation, we co-expressed CFTR with Rab1a(N124I) or Rab2(N119I), dominant negative mutants that are defective in guanine nucleotide binding and interfere with ER to Golgi transport in BHK cells (18, 47). Control experiments demonstrated that the Rab mutants were functionally expressed and blocked VSV-G processing to the endo H-resistant complex Golgi glycoisoform as reported previously (18, 47) (Fig. 2D, upper endo H r bands). In contrast, neither Rab1(N124I) nor Rab2(N119I) affected CFTR processing from the band B to the band C glycoisoform or the CFTR C/B ratio (Fig. 3, A–C). Thus, dominant negative Rab GTPases controlling the function of pre-Golgi transport intermediates required for anterograde or retrograde trafficking between the ER and Golgi (37, 48, 49) did not interfere with CFTR maturation.

**CFTR Maturation Is Independent of Syn5 Function**—Rab GTPases control the assembly of the targeting/fusion machinery involved in ER to Golgi transport (37, 48, 49). This machinery includes members of the syntaxin family of SNARE proteins (50, 51). Syntaxin 5 (Syn5) is specifically required for the fusion of COPII transport vesicles with acceptor Golgi membranes, and overexpression of Syn5 potently inhibits VSV-G ER to Golgi transport (25, 37, 52).

To examine the role of Syn5 in CFTR processing, we co-expressed CFTR with full-length Syn5 and followed the kinetics of CFTR maturation in BHK cells. Similar to dominant negative Arf1 and Rab1a/Rab2 mutants, overexpression of Syn5 had no effect on CFTR maturation to the band C glycoisoform (Fig. 4, A–C) but strongly inhibited the processing of VSV-G (Fig. 4D) in parallel experiments, confirming that Syn5 was functionally expressed (25). The lack of effects of dominant negative Arf or Rab components or overexpression of the Syn5 SNARE component specific for ER to Golgi transport suggests that CFTR trafficking in the early secretory pathway follows a non-conventional pathway in BHK cells.

**Regulation of CFTR Transport by Arf1, Rab1a, and Syn5 Is Cell Type-specific**—Previous studies (9, 53–55) have demonstrated that trafficking of CFTR from post-Golgi regulated secretory compartments to the plasma membrane is cell type-specific. For example, cAMP stimulates trafficking of vesicles containing CFTR to the cell surface in some cell types but not others. Therefore, we examined if CFTR transport in the early secretory pathway was also cell type-specific and universally insensitive to Arf1, Rab1, and Syn5 function. We overexpressed CFTR with Sar1-GTP(H79G), Arf1-GTP(Q71L), Rab1(N124I), or Syn5 in a panel of heterologous cell lines including CHO, HeLa, and 293T cells. In all cell lines examined, CFTR maturation to the band C glycoisoform was dependent on Sar1 function (Fig. 5), indicating that CFTR export from the ER proceeds by a conserved COPII-dependent pathway. However, like the BHK cell line, we found that CFTR maturation in CHO cells was independent of Arf1/Rab1a/Syn5 function (Fig. 5A). The inability of Arf1, Rab1, and Syn5 to inhibit CFTR processing in CHO cells is consistent with the morphological absence of CFTR from Golgi cisternae in this cell line (14). In contrast to the above results, CFTR maturation to the band C glycoisoform in HeLa and 293T cells was dependent on Arf1/Rab1a/Syn5 function (Fig. 5, B and C), as the dominant negative GTPase mutants or overexpressed Syn5 strongly inhibited CFTR maturation. Thus, the mechanism of CFTR transport from the ER to the Golgi is cell type-dependent.

**CFTR Processing Is Dependent on Golgi Function**—Given

![Fig. 3. CFTR processing is independent of Rab1a and Rab2 function in BHK cells. A–C. BHK cells were cotransfected with pcDNA3.1-CFTR and empty vector or with pcDNA3.1-CFTR and pET-Rab1a(N124I) or pET-Rab2(N119I). A, quantitation of CFTR band B. B, representative autoradiogram is shown. C, ratio of CFTR band C to band B plotted as a function of time. CFTR processing was not affected by dominant negative Rab1a(N124I) or Rab2(N119I). D, inhibitory effects of Rab1a(N124I) and Rab2(N119I) on processing of VSV-G from the endo H-sensitive (endo H r) ER form to the endo H-resistant (endo H s) Golgi form.](image-url)
our findings that CFTR trafficking in BHK and CHO cells was independent of Arf1/Rab1/Syn5 function, we next examined if CFTR maturation required a functional Golgi apparatus. To determine whether CFTR utilizes standard processing enzymes required for restructuring N-linked oligosaccharides from mannose-containing glycoisoforms present in the ER to Golgi-processed glycoisoforms containing galactose and sialic acid present in the cis, medial, and trans Golgi compartments, we expressed CFTR in the mutant CHO cell line clone 15B. CHO clone 15B cells lack the medial Golgi-modifying enzyme GlcNAc transferase I and therefore cannot modify N-linked oligosaccharides beyond the Man5 containing cis-Golgi glycoisoform, as has been demonstrated for VSV-G (56–58). Although CFTR was efficiently processed in parental CHO cells, CFTR was not processed to the band C glycoisoform in mutant in CHO-15B cells (Fig. 6A). Thus, CFTR requires a predominantly medial Golgi-localized processing enzyme GlcNAc transferase I for its maturation.

To determine the extent of contribution of other more trans Golgi enzymes to the processing of CFTR, we took advantage of BFA, a fungal metabolite that causes rapid resorption of Golgi cis, medial, and trans-glycosylation enzymes into the ER but not the trans Golgi network (TGN) (59). When the cargo protein VSV-G is held in the ER in the presence of BFA, it partially matures and acquires endo H resistance, confirming that at least cis and medial Golgi enzymes are redistributed to the ER (60) (data not shown). By contrast, and consistent with previous reports (13, 23), CFTR processing from the band B to the band C glycoisoform in transfected BHK cells was completely blocked by BFA (Fig. 6B). This result suggests that glycosylation enzymes located in the distal TGN/endosomal compartments, which are insensitive to BFA-induced collapse into the ER, may be required for maturation of CFTR to the band C glycoisoform.

**CFTR Maturation Is Partially Dependent on a Rab6 Function**—Two distinct pathways regulating Golgi to ER retrograde transport have been described as follows: an early Golgi COPI-dependent and a late Golgi COPI-independent pathway (39, 61). Given our finding that CFTR processing was dependent on
Novel Intracellular Trafficking of CFTR

Golgi function but independent of COPI/Arf1 function, we considered the possibility that CFTR trafficking through the secretory pathway was dependent on the recycling of novel components involved in the late COPI-independent pathway. The COPI-independent pathway is regulated by the Rab6 GTPase that is involved in retrograde transport of various Golgi glycosylation enzymes and movement of bacterial toxins including Shiga toxin/Shiga-like toxin-1 from the endosome through the TGN to the ER (61–63).

To examine the role of Rab6 in CFTR processing, we co-expressed CFTR with wild-type and dominant negative Rab6 proteins and followed the kinetics of CFTR maturation in BHK cells. Overexpression of Rab6 wild-type or Rab6-GDP(T27N), a mutant locked in the inactive GDP-bound state that prevents retrograde trafficking of Golgi glycosylation enzymes and toxins but does not affect Golgi structure (61–63), had no effect on CFTR processing based on the rate of appearance of the band C glycoisoform (Fig. 7, A–C). Thus, CFTR processing is not sensitive to the Rab6-dependent retrograde pathway.

Next, we examined the effects of Rab6-GTP(Q72L), a mutant locked in the active GTP-bound state. Overexpression of Rab6-GTP(Q72L) triggers complete collapse of late Golgi compartments (possibly including the TGN) to the ER and blocks transport to the cell surface (62, 63). If processing to the band C glycoisoform requires enzymes in a Rab6-dependent late Golgi compartment, a prediction is that CFTR retained in the ER under these conditions may become partially modified. Indeed, although no processing could be detected in the presence of BFA (Fig. 6B), we observed partial processing of CFTR to the band C glycoisoform in the presence of the Rab6-GTP(Q72L) mutant (Fig. 7, A–C). Thus, the complete collapse of Golgi elements induced by the Rab6 GTP-restricted mutant into the ER suggests that in addition to early Golgi-associated processing enzymes, late Golgi and TGN-associated Golgi proteins participate in CFTR processing in the non-conventional pathway.

CFTR Maturation Is Dependent on Syntaxin 13 Function—CFTR processing was dependent on Golgi function in BHK and CHO cells displaying the non-conventional pathway. Therefore, we considered the possibility that the non-conventional CFTR trafficking pathway in BHK cells results in an initial direct delivery of CFTR to the trans Golgi network/endosomal system (64, 65).

To test the above possibility, we examined the role of several syntaxins involved in regulating transport at other steps of the exocytic and endocytic pathways. We initially focused on syntaxin 1 (Syn1) and syntaxin 8 (Syn8). Syn1 is currently recognized to facilitate targeting and fusion of vesicles to the cell surface (50). Syn1 directly interacts with the CFTR N terminus and inhibits both CFTR function and cAMP-stimulated CFTR trafficking from post-Golgi organelles to the plasma membrane (66–69). Syn8, by contrast, is preferentially associated with the early endosome (70, 71) and interacts with the regulatory domain of CFTR, although the functional significance of this interaction remains unknown (72). Overexpression of either Syn1 or Syn8 had no measurable effect on CFTR maturation from the immature band B to the mature band C glycoisoform (data not shown), suggesting that these SNARE proteins do not modulate CFTR trafficking in the early secretory pathway.

We next examined the possible involvement of syntaxin 13 (Syn13). Syn13 localizes to both early and late recycling endo-
somes and regulates transferrin receptor recycling to a juxta-
TGN endosomal compartment (73–75). Strikingly, overexpression of full-length Syn13 completely blocked CFTR processing to the mature band C glycoisoform in BHK cells (Fig. 8, A–C). Moreover, unlike the block imposed by Sar1-GTP(H79G), overexpression of Syn13 did not stabilize CFTR band B against degradation (Fig. 8D), emphasizing the importance of the CO-
PPI machinery in these events (Fig. 1). Whereas the CFTR C/B band ratio was ∼4 in control cells, the ratio fell nearly 10-fold to ∼0.4 in cells overexpressing Syn13 after a 3-h chase period (Fig. 8C). Syn13 inhibition was dose-dependent as the general level of expression correlated with the extent of block of CFTR processing from the band B to the band C glycoisoform (data not shown). Importantly, Syn13 had no effect on VSV-G processing to the endo H-resistant Golgi glycoisoform in parallel experiments (Fig. 8D), indicating that Syn13 inhibition of CFTR processing was specific for the non-conventional pathway.

Collectively, our data suggest that CFTR maturation through the non-conventional pathway in BHK cells is achieved via initial trafficking of CFTR through an Arf1/Rab1/Syn5-independent pathway that may involve the late Golgi/ endosomal system, followed by a Syn13-dependent recycling pathway through the Golgi where CFTR acquires complex carbohydrates and is processed from the immature band B to the mature band C glycoisoform.

**DISCUSSION**

**Transport of CFTR through the Early Secretory Can Utilize a Non-conventional Pathway**—We have described for the first time the intracellular trafficking of CFTR through the early secretory pathway at the molecular level. In all cell lines examined, CFTR maturation from the immature band B to the mature band C glycoisoform was blocked by co-expression of dominant negative Sar1-GTP(H79G). Thus, CFTR export from the ER proceeds by an evolutionarily conserved COPII-mediated pathway. However, following ER export, the mechanism of CFTR maturation was critically dependent on cell type. In BHK and CHO cells, CFTR processing could bypass the requirement for Arf1, Rab1, and Syn5, components necessary for ER to Golgi transport of other cargo proteins (18, 24, 25, 28, 52). This non-conventional pathway, however, was sensitive to overexpression of Syn13, a transport component that does not affect transport of VSV-G through the Golgi via the conventional pathway. On the other hand, in both HeLa and 293T cells, CFTR processing to the band C glycoisoform was sensitive to Arf1/Rab1/Syn5. Given that all cell lines tested house the conventional pathway (as indicated by the effect of overexpression of dominant negative transport components on the trafficking of VSV-G), both BHK and CHO cells must have an uncharacterized post-ER pathway that can be utilized by CFTR for movement to the Golgi. A limitation of our results is that we have been unable to transfect efficiently polarized epithelial cell lines that express CFTR using the vaccinia transient expression system. Nevertheless, this system has the advantage of allowing us to monitor effects of lethal dominant negative Sar1, Arf1, and Rab1 mutants (<5 h) prior to more general effects on cell metabolism (26, 43). Conventional transient expression systems requiring 24–48 h of incubation to obtain sufficient levels of each mutant to inhibit transport may have secondary effects that would be difficult to evaluate. However, our results are highly correlative with previous morphological studies that showed in situ that in CFTR-expressing polarized cells, CFTR is absent from the Golgi (14), providing a foundation for the current experiments (see below).

One possibility to explain our results is that during export from the ER, cargo molecules such as CFTR and VSV-G can be segregated into distinct classes of COPII carriers with novel destinations (76). Consistent with this possibility, multiple isoforms of the COPII Sec33/24 selection machinery are found in both yeast and mammalian cells, and several lines of evidence suggest that they affect selection of different types of cargo (76–80). Alternatively, following export from the ER using common COPII machinery components, CFTR is segregated from the Arf1/Rab1/Syn5 pathway during the maturation of pre-Golgi intermediates and moves via a more direct pathway to the TGN/endosomal system. The fact we can detect CFTR maturation via both Arf1/Rab1/Syn5-dependent and independent pathways indicates that the inability of Arf1/Rab1/Syn5 mutants to inhibit CFTR transport in BHK and CHO cells is not simply a consequence of the relatively slow and inefficient transport properties of wild-type CFTR. Moreover, the fact that transport of VSV-G in all cell lines tested was inhibited by Arf1/Rab1/Syn5 suggests that the non-conventional mechanism is utilized in the presence of the conventional pathway.

**The Non-conventional Pathway May Target CFTR to a Late Golgi/Endosomal Compartment**—The possibility that CFTR is directly transported from the ER or VTCs to a late Golgi/TGN/ endosomal compartment via an Arf1-, Rab1-, and Syn5-independent pathway is consistent with the absence of CFTR from
the early and central Golgi cisternae of the Golgi stack in CHO cells and in epithelial tissues using morphological approaches (14). A requirement for TGN-localized processing enzymes came from the observation that although collapse of the cis, medial, and trans Golgi compartments to the ER by BFA was not sufficient to support maturation of CFTR, overexpression of Rab6-GTP(Q72L) (which causes collapse of all Golgi compartments, potentially including the TGN) (62) was required to detect partial processing of CFTR from the band B to the band C glycoisoform.

Evidence for a more direct pathway from the ER to the TGN stemmed from the observation of overexpression of Syn13 potently inhibited CFTR processing to the band C glycoisoform in BHK cells. In contrast, overexpression of Syn8 or Syn1, syntaxins that bind CFTR (68, 72), had no effect on CFTR maturation. The lack of effect of Syn1 is consistent with the recent report (67) that Syn1 preferentially binds the mature band C glycoisoform of CFTR in vitro. Thus, Syn1 interaction with CFTR may occur only following CFTR maturation. This interaction may be required for CFTR cycling between endosomal and plasma membrane pools and/or for regulation of surface CFTR function (66–68).

Although the precise function of Syn13 in exocytic or endocytic trafficking is unknown, one possibility is that Syn13 may physically interact with CFTR. In preliminary experiments, we have been unable to demonstrate an interaction between Syn13 and CFTR using conditions identical to those used to observe the interaction of CFTR with Syn1 (68, 72). A second possibility is that Syn13 may regulate the targeting and fusion of ER-derived transport intermediates containing CFTR to a late Golgi (TGN) or endosomal compartment. Alternatively, we cannot exclude the possibility that Syn13 may control the recycling of a critical component between the endosomes and the TGN that is required for CFTR processing from the band B to the band C glycoisoform. Although Syn13 binds EEA1, a Rab5-GTP effector protein involved in early endosome function (73, 81), we have been unable to demonstrate a dominant negative effect of Rab5 mutants on CFTR processing to the band C glycoisoform.2

The potential existence of a direct, non-conventional pathway of CFTR transport from the ER to terminal Golgi compartments is consistent with the recent observations (82) of a close apposition between the ER and the trans-Golgi cisternae by high resolution three-dimensional tomography. However, the use of such a pathway raises a conundrum. Processing of N-linked oligosaccharides such as those found on CFTR occurs through defined, sequential steps by processing enzymes in the Golgi that are distributed along the cis to trans axis. Thus, once delivered to the TGN/endoendosome it would be necessary for CFTR to recycle back to the cis compartment to initiate processing, a result inconsistent with morphological analyses (14). Alternatively, given that the distribution of Golgi processing enzymes occurs as a gradient (27), it remains possible that processing of CFTR is initiated by low levels of early processing enzymes present in the TGN. Although it is unclear why CFTR would utilize such a non-conventional approach to maturation, one possibility is that the presence of high levels of CFTR in Golgi compartments could disrupt Golgi function.

Is CFTR Alone in the Use of a Non-conventional Pathway?—
Alternative trafficking pathways from the ER, which may initially bypass the Golgi apparatus, have been reported previously (83–91) for cholesterol, plant vacuolar proteinases, the AE1 anion exchanger, and numerous viral proteins. AE1, for example, may be directly transported from the ER to the plasma membrane where it initially contains oligosaccharides sensitive to digestion by endoglycosidase F. Following endocytosis, AE1 recycles to Golgi membranes, where it acquires complex carbohydrates containing N-acetyllactosamine composed of Galβ1–4GlcNAcβ1–3 disaccharides that confer resistance to endoglycosidase F (88). Interestingly, the mature CFTR band C oligosaccharides also contain repeating units of N-acetyllactosamine that are distinct from the conventional complex sugars found on N-linked oligosaccharides of SV5-G that contain terminal sialic acid residues (31, 92), again consistent with a non-conventional CFTR trafficking pathway.

Effects on CFTR Stability—One unanticipated and important finding was that overexpression of Sar1-GTP(H79G), which stabilizes COPII coats on ER-derived export sites and COPII vesicles, stabilized immature wild-type CFTR band B and decreased degradation by −3−6-fold. By contrast, overexpression of Sar1-GTP(H79G) did not stabilize the degradation rate of CFTRΔF508.2 Combined with the low level of co-localization of ΔF508 with either Sar1 or Sec23 in vivo when compared with wild-type CFTR (Fig. 1E), these data suggest that wild-type CFTR, but not CFTRΔF508, may stably interact with components of the COPII export machinery to divert CFTR away from ER quality control systems that normally scan the ER for misfolded CFTR (10, 11, 93, 94). We propose that cargo capture by the COPII machinery could represent a key defect in the difference between export of wild-type and CFTRΔF508 from the ER and are currently exploring the identity of the components found in CFTR prebudding ER complexes (34) that lead to the export of the wild-type but not the CFTRΔF508 mutant.

In contrast to the effects of Sar1 on promoting stability of wild-type CFTR in the ER, preventing COPI coat disassembly from pre-Golgi intermediates by overexpression of the dominant negative Arf1-GTP(Q71L) mutant had no effect on the maturation or stability of wild-type or CFTRΔF508 in all cells tested.2 Because Arf1-GTP(Q71L) largely prevents recycling of cargo, particularly molecules containing C-terminal KX(K)X motifs, from pre-Golgi intermediates back to the ER (95), the need for retrieval of CFTR through COPII-dependent intermediates is unlikely to be the mechanism involved in delivery of CFTR to the degradative machinery. However, this does not rule out the possibility of a COPI-independent mechanism involving an uncharacterized retrieval pathway involving diphosphatidylinositol phospholipid motifs (96). This is unlikely to involve other Arf-dependent events as other Arf family members, including Arf3-GTP(Q71L), Arf4-GTP(Q71L), Arf5-GTP(Q71L), and Arf6-GTP(Q71L), were also tested and found not to inhibit CFTR processing or degradation.2

Implication for CF Disease—Our findings on the molecular mechanism(s) of CFTR trafficking through the early secretory pathway have important implications for treatment of individuals with CF. Although the specific trafficking route(s) utilized by CFTR in the early secretory pathway of polarized epithelial cells in the lung and other tissues remains unknown, our previous work demonstrating that CFTR is not readily detectable in Golgi cisternae, but is readily detected in the ER, plasma membrane, and endosomal compartments of duodenal crypt and salivary duct cells, suggests that the non-conventional pathway predominates in epithelia in situ (14). Because the trafficking of CFTR through the non-conventional pathway may be restricted to a limited group of proteins, therapeutic approaches directed toward augmenting this specialized pathway to promote transport of CFTRΔF508 in CF patients may be specific and not impact the transport of other proteins required for cell differentiation and growth.

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2 J.-S. Yoo and W. E. Balch, unpublished observations.
