The EMBO Journal vol.13 no.24 pp.6076–6086, 1994

Conformational maturation of CFTR but not its mutant counterpart (ΔF508) occurs in the endoplasmic reticulum and requires ATP

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Communicated by E.Neher

Metabolic labeling experiments followed by immunoprecipitation were performed to investigate the kinetics, location and inhibitor sensitivity of degradation of both wild-type (wt) and mutant (ΔF508) cystic fibrosis conductance transmembrane regulator (CFTR). At the earliest stages of the biosynthetic process, both wt and ΔF508 CFTR were found to be susceptible to degradation by endogenous proteases. Virtually all ΔF508 CFTR and 45–80% of wt CFTR were rapidly degraded with a similar half-life ($t_{\frac{1}{2}} \approx$ 0.5 h). The remaining wt CFTR attained a protease-resistant configuration regardless of whether traffic between the endoplasmic reticulum (ER) and Golgi was operational. Metabolic energy is required for the conformational transition, but not to maintain the stability of the protease-resistant wt CFTR. Intracellular degradation of ΔF508 CFTR and of incompletely folded wt CFTR occurs in a non-lysosomal, pre-Golgi compartment, as indicated by the sensitivity of proteolysis to different inhibitors and temperature. Accordingly, products of the degradation of ΔF508 CFTR could be detected by immunoblotting in isolated ER, but not in the Golgi. Together, these results suggest a dynamic equilibrium between two forms of wt CFTR in the ER: an incompletely folded, protease-sensitive form which is partially converted by an ATP-dependent process to a more mature form that is protease-resistant and capable of leaving the ER. The inability ΔF508 CFTR to undergo such a transition renders it susceptible to complete and rapid degradation in a pre-Golgi compartment.

Key words: cystic fibrosis transmembrane conductance regulator/post-translational modification/protein folding/proteolysis

Introduction

Cystic fibrosis (CF), a common inherited disorder in the Caucasian population, is characterized by impaired epithelial salt and water transport (see Quinton, 1990; Welsh and Smith, 1993; Riordan, 1993, for reviews). The disease is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989). The product of the CF gene has been detected in surface and endosomal membranes, where it functions as a protein kinase A (PKA)- and ATP-regulated Cl^- channel (Anderson *et al.*, 1991; Drumm *et al.*, 1991; Tabcharani *et al.*, 1991; Bear *et al.*, 1992; Lukacs *et al.*, 1992; Biwersi and Verkman, 1994).

The most prevalent mutation in the CF population, found in ≥90% of patients, is the deletion of phenylalanine at position 508 (ΔF508 CFTR) (Tsui, 1992; Sferra and Collins, 1993). Loss of this residue, which is located in the first nucleotide binding domain (NBD1) of CFTR, precludes the activation of Cl^- permeability by PKA in several epithelia (Quinton, 1990). This defect is not due to the inability of the mutant gene product to transport Cl^-, since in heterologous expression (Drumm *et al.*, 1991; Denning *et al.*, 1992a; Lukacs *et al.*, 1993a) and reconstitution experiments (Li *et al.*, 1993) ΔF508 CFTR was found to display near normal Cl^- channel activity. Instead, available evidence suggests that the defective Cl^- permeability results from the inability of ΔF508 CFTR to reach the plasma membrane. Briefly, unlike wild-type CFTR (wt CFTR), which acquires complex type, *N*-linked oligosaccharides in the medial Golgi, ΔF508 CFTR remains core-glycosylated, indicating its retention in an earlier compartment (Cheng *et al.*, 1990). Moreover, immunolocalization studies at the light and electron microscopic level failed to detect ΔF508 CFTR in the plasma membrane of epithelial cells from patients homozygous for ΔF508 CFTR (Denning *et al.*, 1992b; Engelhardt *et al.*, 1992; Kartner *et al.*, 1992) and in transfected cells (Yang *et al.*, 1993). Instead, the mutant protein was proposed to be associated with the endoplasmic reticulum (ER) (Cheng *et al.*, 1990; Yang *et al.*, 1993). Together, these observations strongly suggest that mislocalization of ΔF508 CFTR is the primary cause of the defective plasma membrane Cl^- permeability in CF.

Abnormal processing of the mutant CFTR is probably the consequence of its structural misfolding, as suggested by: (i) the alleviation of the processing defect by reduced temperatures (Denning *et al.*, 1992a; Lukacs *et al.*, 1993a); (ii) the reduced stability of ΔF508 CFTR at the plasma membrane and of a synthetic peptide representing a portion of the NBD1 which comprises the ΔF508 mutation (Lukacs *et al.*, 1993a; Thomas *et al.*, 1992a,b); (iii) the generation of functional revertants by point mutations in other loci of NBD1 (Teem *et al.*, 1993). Improperly folded ΔF508 CFTR may be unable to reach the surface membrane due to retention and/or accelerated degradation in endomembranes. Yet, although the available morphological and biochemical data are consistent with the notion that ΔF508 CFTR is trapped in the ER, neither the location nor the kinetics of degradation of the mutant molecules have been

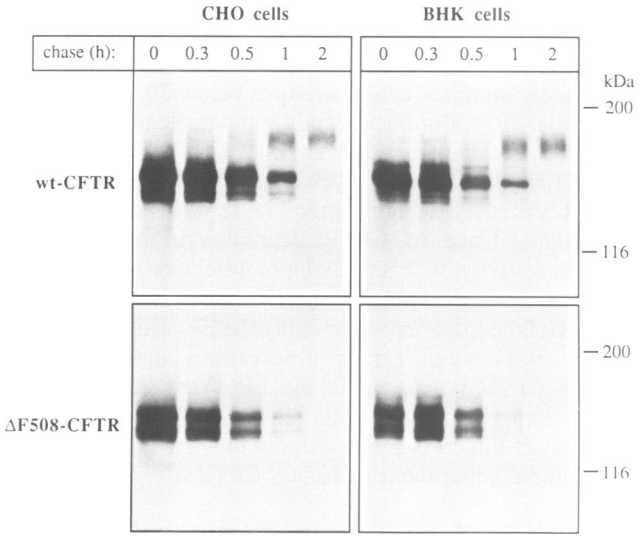

Fig. 1. Analysis of the biogenesis and degradation of wt CFTR and ΔF508 CFTR in transfected cells. CHO cells (left column) or BHK cells (right column) stably transfected with either wt CFTR (top) or ΔF508 CFTR (bottom) were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (0.14–0.3 mCi/ml) for 15 min and chased in αMEM supplemented with 0.75 mM cold methionine for the indicated time (h). The cells were next lyzed and CFTR was immunoprecipitated with a mixture of the M3A7 and L12B4 monoclonal antibodies (1.2 μg/ml each) and analyzed by SDS–PAGE and fluorography. The fluorogram is representative of 2–5 experiments. The position of molecular weight markers is indicated on the right.

defined. The purpose of the present experiments was therefore to compare the rates of degradation of newly synthesized wt and ΔF508 CFTR and to identify the compartment where degradation of ΔF508 CFTR occurs. A sizable fraction of the wt CFTR is also rapidly degraded without ever reaching the plasmalemma. The site and kinetics of degradation of this incompletely folded wt CFTR appear to be similar to those of ΔF508 CFTR. In addition, we found that an ATP-dependent process is essential to the proper folding of the core-glycosylated wt CFTR, enabling it to attain protease resistance and to exit the ER *en route* to the plasma membrane.

Results

Turnover rates of immature wt CFTR and ΔF508 CFTR

The inability of ΔF508 CFTR to reach the plasma membrane could, in principle, result from an accelerated rate of degradation. To assess this possibility, we compared the stability of newly synthesized wt and mutant CFTR stably expressed in CHO or BHK cells. After pulse-labeling the transfected cells for 15 min with [^{35}S]-methionine and [^{35}S]cysteine, CFTR was immunoprecipitated and analyzed by SDS–PAGE (Figure 1). As reported earlier (Pind *et al.*, 1994), newly synthesized wt CFTR (0 min chase) migrates as multiple bands of 130–150 kDa in both transfectants. This has been identified as the endo H-sensitive, core-glycosylated form of CFTR, also known as the B form (Cheng *et al.*, 1990; Pind *et al.*, 1994). The source of the multiple forms of core-glycosylated wt CFTR has been discussed in detail elsewhere (Pind *et al.*, 1994). Following the chase, the core-glycosylated wt CFTR disappears gradually, with a $t_{\frac{1}{2}}$ of 30–40 min. Concurrently, a fraction of the labeled protein is converted to a species with a higher molecular mass (170–180 kDa). This has been identified as the complex-glycosylated, endo H-resistant or C form of CFTR (Cheng *et al.*, 1990; Pind *et al.*, 1994). It is noteworthy that a sizable fraction (≥80%) of wt CFTR fails to mature and is conceivably degraded by rapid proteolysis, since neither secretion nor insoluble aggregate formation could be detected (not shown). Importantly, the combination of monoclonal antibodies used (M3A7 and L12B4; Kartner *et al.*, 1992) effectively immunoprecipitated both forms of wt CFTR (Pind *et al.*, 1994).

The finding that a large fraction of immature wt CFTR is unable to mature and seemingly becomes rapidly degraded may be the result of heterologous expression in non-epithelial cells. To test this possibility, degradation rates and maturation efficiency were also estimated in the epithelial lines T84 and Calu-3, which express CFTR endogenously. As shown in Figure 2, disappearance of the core-glycosylated CFTR also occurred rapidly in these cells ($t_{\frac{1}{2}} \approx 50$ min). Approximately 50% of the labelled wt CFTR failed to mature in these epithelial lines and disappeared with a calculated $t_{\frac{1}{2}}$ of 30–40 min (see below).

As illustrated in Figure 1, the pulse-labeled core-glycosylated ΔF508 CFTR could not be processed to the complex-glycosylated species and, in agreement with earlier observations (Cheng *et al.*, 1990; Denning *et al.*, 1992a; Marshall *et al.*, 1994; Pind *et al.*, 1994), almost completely disappeared after 2 h. Multiple pulse-chase experiments indicated that the $t_{\frac{1}{2}}$ for ΔF508 CFTR was only slightly shorter than that of the wt protein in CHO cells, but the converse was true in BHK cells (Figure 2). These observations suggest that the ΔF508 mutation does not greatly alter the susceptibilty of the newly synthesized protein to degradation.

Degradation of the core-glycosylated wt CFTR and ΔF508 CFTR occurs in a non-lysosomal compartment

To delineate the biochemical processes and identify the intracellular organelle(s) responsible for the extensive breakdown of core-glycosylated wt CFTR and the complete degradation of ΔF508 CFTR, we determined the effects of proteolysis inhibitors with well-defined sites of action. The results are summarized in Table I. Inhibition of endo-lysosomal proteolysis by dissipation of the acidic intraluminal pH with lysosomotropic agents (ammonium chloride and chloroquine) or with the ionophore monensin did not substantially retard the degradation of either wt CFTR or ΔF508 CFTR. Similarlly, bafilomycin B, an inhibitor of the vacuolar H pump, did not alter the degradation of CFTR (data not shown). That the weak bases and monensin effectively dissipated the pH gradient across the membrane of acidic organelles was ascertained by monitoring the fluorescence of acridine orange (data not shown). These findings suggest that the endo-lysosomal compartment does not participate in the degradation of CFTR. Consistent with this notion, leupeptin and pepstatin, inhibitors of lysosomal cathepsins B, D, H and L (Holtzman, 1989), failed to interfere with the degradation of core-glycosylated wt CFTR or ΔF508 CFTR, even

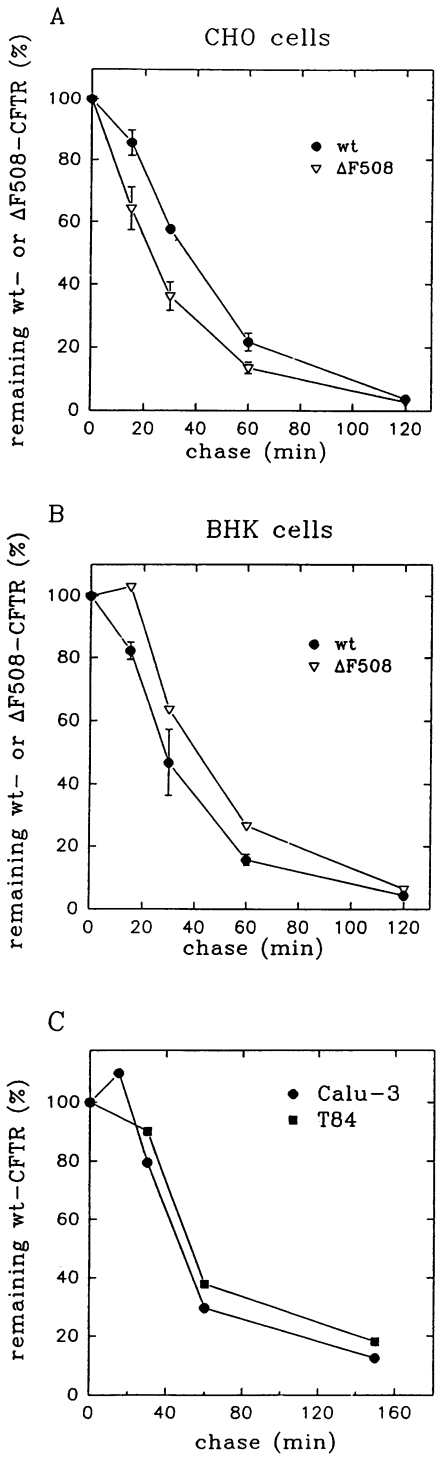

Fig. 2. Rate of disappearance of core-glycosylated CFTR. Cells were pulse-labeled for 15 min and chased for the indicated time. CFTR was then immunoprecipitated and subjected to SDS–PAGE as described in the legend to Figure 1. The amount of core-glycosylated wt CFTR (solid symbols) or ΔF508 CFTR (open symbols) was quantified by phosphorimage analysis. The amount of core-glycosylated CFTR remaining after the indicated chase periods is expressed as a percentage of the initial label. Data are means of 2–5 independent experiments (except for T84, where $n = 1$). Error bars indicating ± 1 SEM are included where $n \geq 3$. Since the turnover rates of the individual bands that constitute the core-glycosylated wt CFTR and ΔF508 CFTR were indistinguishable, they were quantified jointly. (**A**) CHO cells; (**B**) BHK cells; (**C**) Calu-3 (circles) and T84 (squares) cells.

when cells were preincubated with the inhibitors for 14 h to ensure delivery to intracellular compartments (Table I).

Protein degradation in pre-Golgi compartments is reportedly inhibited at temperatures below 30°C (Klausner and Sitia, 1990; Bonifacino and Lipincott-Schwatrz, 1991). When pulse-labeled cells were chased at 16°C, ~50% of the initially labeled core-glycosylated wt CFTR and ΔF508 CFTR was still detectable after 3 h (Table I). The temperature dependence of CFTR degradation, together with its insensitivity to lysosomal protease inhibitors and to lysosomotropic agents, suggest that breakdown occurs in a pre-Golgi compartment, possibly the ER. This conclusion is consistent with earlier observations indicating that ΔF508 CFTR is trapped in the ER (Yang *et al.*, 1993). More importantly, our data also suggest that a large fraction of the core-glycosylated wt CFTR is degraded in the same compartment as ΔF508 CFTR.

Proteolysis of the core-glycosylated wt CFTR and ΔF508 CFTR proceeds after disruption of vesicular transport between ER and Golgi

To obtain more direct evidence for the role of the ER in CFTR catabolism, we assessed the effect of inhibitors of intracellular membrane transport on the rate of CFTR degradation. We reasoned that if proteolysis of the core-glycosylated form persists after disruption of vesicular traffic between the ER and Golgi, degradation conceivably occurrs within the former compartment. Two separate means were used to impair translocation of proteins from the ER to the Golgi: depletion of the cytosol by mechanical perforation of the plasma membrane (Beckers *et al.*, 1987) or exposure of the cells to brefeldin A (BFA; Klausner *et al.*, 1992).

Mechanically perforated (semi-intact) cells have been used previously to demonstrate the occurrence of protein degradation in the ER (Stafford and Bonifacino 1991; Wikstrom and Lodish, 1992). A similar protocol was employed here to monitor the fate of pulse-labeled CFTR. As shown in Figure 3, the breakdown of core-glycosylated wt CFTR and ΔF508 CFTR was not prevented by the permeabilization procedure. In fact, the rate of degradation was accelerated under these conditions. Only 12% of the pulse-labeled wt CFTR (lane 3, Figure 3) and 6.7% of ΔF508 CFTR (lane 13, Figure 3) could be detected after a 0.5 h incubation at 37°C in semi-intact cells. In comparison, ≈50% of the pulse-labeled wt CFTR or ΔF508 CFTR remained in intact cells over a comparable period of time (e.g. Figure 2). Permeabilization induced only marginal changes in the content of core-glycosylated CFTR if the cells were kept at 4°C (lanes 2 and 11, Figure 3) and did not accelerate the disappearance of the complex-glycosylated wt CFTR at either 4 or 37°C (lanes 6 and 7). The latter observation indicates that maturation of wt CFTR increases its stability.

The fungal metabolite BFA was used as an alternative and complementary approach to block vesicular transport between the ER and Golgi. Addition of BFA is known to induce disassembly of the Golgi complex, resulting in nearly instantaneous inhibition of protein secretion (Misumi *et al.*, 1986; Klausner *et al.*,1992). The efficacy of BFA on CFTR-expressing CHO cells was first established by assessing the localization of the proximal Golgi marker α-mannosidase II by indirect immuno-

Table I. Inhibitor sensitivity profile of the degradation of core-glycosylated wt CFTR and ΔF508 CFTR in transfected CHO cells

Treatment	Duration of chase (h)	Remaining pulse-labeled core-glycosylated (%)		*n* (wt/ΔF508)
		wt CFTR	ΔF508 CFTR	
None	1	12.0	10.1	3/3
NH_4Cl (10 mM)	1	7.0	7.2	3/3
Monensin (10 μM)	1	7.8	6.3	2/2
Chloroquine (0.5 mM)	1	14.6	12.4	2/2
Leupeptin + pepstatin (10 μg/ml)	1	12.0	13.2	2/2
16°C	3	59.0	49.2	2/2
Cycloheximide (0.5 mM)	1	15.0	14.8	2/3
TPCK (100 μg/ml)	1	3.3	4.2	2/3
TLCK (100 μg/ml)	1	11.4	8.1	2/3
ZPCK (20 μg/ml)	1	2.4	2.4	2/2
ALLM (100 μg/ml)	1	13.0	10.2	2/3
ALLN (100 μg/ml)	1	20.6	24.0	3/4
Diamide (0.5 mM)	1	8.2	12.0	2/3

Pulse labeled (20 min) cells were chased in the presence of the inhibitor for 1 h and processed for quantitation by phosphorimaging as described for Figure 1. To ensure that leupeptin and pepstatin accumulate intracellularly, cells were preincubated overnight and labeled in the presence of the drugs (10 μg/ml). When cells were incubated at 16°C the medium was supplemented with 15 mM HEPES (pH 7.3). The amount of radioactivity associated with CFTR remaining after the chase period is expressed as a percentage of the initial pulse label. Abbreviations: ALLM, *N*-acetyl-leucyl-leucyl-methioninal; ALLM, *N*-acetyl-leucyl-leucyl-norleucinal; TPCK, *N*-tosyl-L-phenylalanine chlormethyl ketone; TLCK, *N*-tosyl-L-lysine chlormethyl ketone; ZPCK, *N*-carbobenzoxy-L-phenylalanine chlormethyl ketone.

fluorescence (Lipincott-Schwarz *et al.*, 1990; Moremen and Robbins, 1991). To assess the effect of BFA on CFTR degradation, pulse-labeled cells were chased in the presence of the fungal metabolite and the radioactivity associated with CFTR was determined. Typical results are illustrated in Figure 4A and quantitative evaluation of two experiments is summarized in Figure 4B. As expected, the conversion of the high-mannose, *N*-linked oligosaccharide form of CFTR to its complex form was completely precluded, consistent with the notion that BFA impairs traffic between the ER and Golgi. More interestingly, BFA was found to have a bimodal effect on the disappearance of the core-glycosylated wt CFTR. Approximately 70–80% of the pulse-labeled protein disappeared at a rate similar to that of untreated cells (Figure 4B). However, a small fraction of the core-glycosylated wt CFTR became protease-resistant, remaining undegraded in the ER for periods of at least 4 h (Figure 4A and B). The nature of this unique form of wt CFTR is discussed in more detail below.

BFA had no effect on the intracellular processing of ΔF508 CFTR in CHO (Figure 4A and C) or in BHK cells (data not shown), suggesting that degradation of the latter takes place in a pre-Golgi compartment. Similar results were obtained when BFA was present during the pulse-labeling step (not shown), excluding the possibility that ΔF508 CFTR is rapidly translocated to a post-ER comparmtent before the inhibitor exerts its effect on membrane traffic. Together, the results of these experiments are consistent with the notion that a sizable fraction of the core-glycosylated wt CFTR and all the ΔF508 CFTR are degraded in a pre-Golgi compartment, probably the ER.

Detection of immunoreactive degradation intermediates of ΔF508 CFTR in isolated ER

Identification of proteolytic breakdown products of CFTR in the ER would provide convincing proof that degradation is indeed occurring in that compartment. To assess this possibility, the post-nuclear supernatant of CHO cells expressing ΔF508 CFTR were subjected to centrifugation

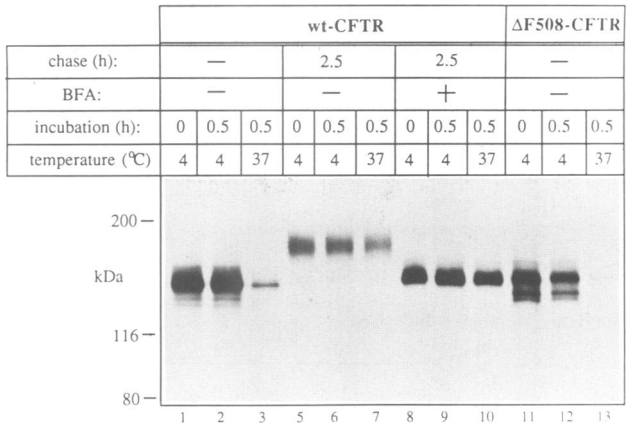

Fig. 3. Stability of wt CFTR and ΔF508 CFTR in 'semi-intact' cells. Subconfluent dishes of wt CFTR- or ΔF508 CFTR-expressing CHO cells were labeled metabolically with [^{35}S]methionine and [^{35}S]cysteine for 20 min and perforated either immediately or after 2.5 h, as described in Materials and methods. When indicated BFA (10 μg/ml) was present during the pulse-labeling and the chase as well. The perforated cells were resuspended in 50 mM potassium acetate, 25 mM HEPES, 2.5 mM magnesium acetate, 1.8 mM $CaCl_2$ and 5 mM EGTA, pH 7.2. Identical numbers of cells were then incubated for the indicated times at 4 or 37°C. Finally, labeled wt CFTR (lanes 1–10) and ΔF508 CFTR (lanes 11–13) were analyzed by fluorography after immunoprecipitation and SDS–PAGE, as in Figure 1. Representative of two experiments.

on discontinuous sucrose gradients, to isolate ER- and Golgi-enriched fractions (Bole *et al.*, 1986). As illustrated in Figure 5A, while the ER fraction was virtually devoid of contamination by other organelles, a partial overlap existed between the Golgi and plasma membrane fractions, as indicated by the distribution of the respective markers α-mannosidase II and alkaline phosphatase.

The presence of ΔF508 CFTR and its degradation products was assessed in the fractions displaying highest enzyme marker activity and minimum contamination by immunoblotting with the M3A7 monoclonal anti-CFTR antibody (Kartner *et al.*, 1992). As illustrated in Figure

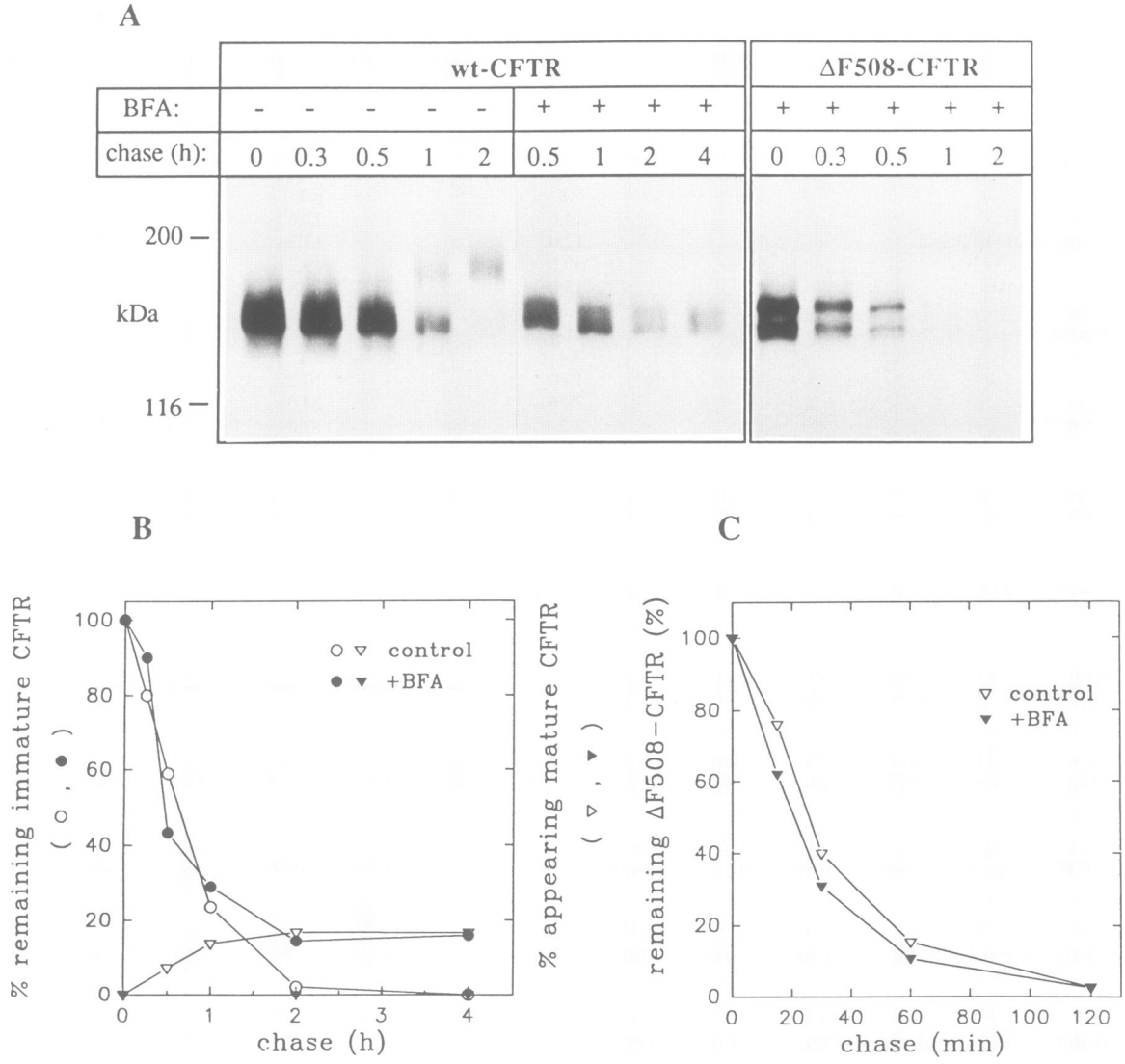

Fig. 4. Effect of BFA on the turnover of pulse-labeled wt CFTR and ΔF508 CFTR. CHO cells expressing either wt CFTR or ΔF508 CFTR were pulse-labeled as before and chased either in the presence (+) or absence (−) of BFA (10 μg/ml) for the indicated time. The wild-type and mutant CFTR were then analyzed by immunoprecipitation and SDS−PAGE, as in Figure 1. (**A**) Representative fluorograms. (**B**−**C**) Mean values from two similar experiments quantitated by phosphorimage analysis. Open symbols, untreated cells; solid symbols, BFA-treated cells. (B) Circles, percent remaining immature (core-glycosylated) CFTR; Triangles, appearance of mature (complex-glycosylated) CFTR as a percentage of initially labeled (immature) protein. (C) Triangles, remaining ΔF508 CFTR as a percentage of the initial label.

5A, virtually all the immunoreactive ΔF508 CFTR was found in the ER fraction. Consistent with earlier morphological and functional data, ΔF508 CFTR was not detectable in the plasma membrane (Denning *et al.*, 1992a; Engelhardt *et al.*, 1992; Kartner *et al.*, 1992; Yang *et al.*, 1993). However, trace amounts of the mutant protein were found in the Golgi-enriched fractions. This may reflect contamination of the Golgi fraction with ER vesicles or may instead indicate that some of the ΔF508 CFTR in fact reaches the Golgi, where it may either be retrieved by the ER or degraded, as has been reported for other misfolded or unassembled proteins (Pelham, 1989; Hsu *et al.*, 1991; Amitay *et al.*, 1992; Hammond and Helenius, 1994).

In cells incubated at 37°C, degradation intermediates of ΔF508 CFTR were hardly detectable. This may imply that the C-terminal epitope recognized by the M3A7 antibody (Kartner *et al.*, 1992) is released to the cytosol. Alternatively, proteolysis may occur rapidly and thoroughly, yielding small fragments that are not detectable by our immunoblotting protocol. To analyze the latter possibility, proteolysis was partially inhibited by keeping the cells at reduced temperature for 24 h. When probed with the M3A7 antibody, subcellular fractions of such cells revealed the presence of immunoreactive polypeptides smaller than ΔF508 CFTR, probably its proteolytic fragments. These fragments were detectable in the ER, but not in the Golgi and plasmalemmal fractions (Figure 5B). These observations provide confirmatory evidence that ΔF508 CFTR degradation occurs, at least in part, in the ER.

A fraction of the core-glycosylated wt CFTR attains protease resistance and transport competence in the ER

As discussed above, disruption of vesicular transport from the ER to the Golgi by BFA promoted the appearance of

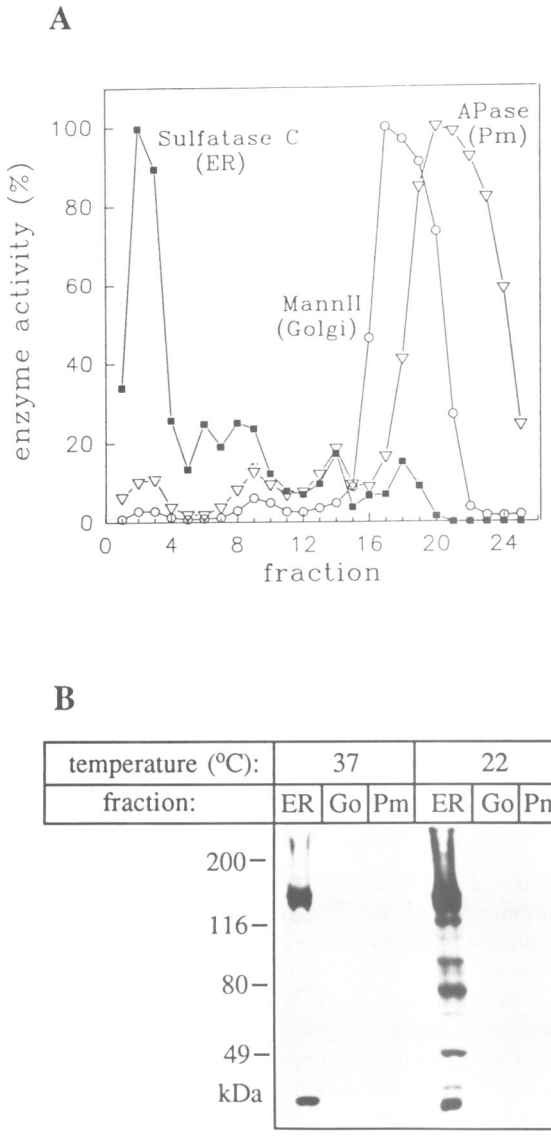

Fig. 5. Detection of intact and partially degraded ΔF508 CFTR in isolated ER. (**A**) ΔF508 CFTR-expressing CHO cells were homogenized, subjected to differential centrifugation and the post-nuclear supernatant was sedimented through a discontinuous sucrose gradient. Fractions were collected from the bottom (fraction 1) and analyzed for the activity of specific markers of the ER (sulfatase C), Golgi (α-mannosidase II, MannII) and plasma membrane (alkaline phosphatase, APase). Enzyme activities are expressed as a percentage of the maximum. A similar distribution of enzyme activities was observed after fractionation of cells grown at 22°C (not shown). (**B**) The subcellular localization of ΔF508 CFTR and its degradation products was analyzed by immunoblotting of ER-, Golgi- (Go) and plasma membrane (Pm)-enriched fractions (fractions 3, 17 and 23 respectively) isolated from cells grown at either 37 or 22°C, as specified. Identical amounts of protein (25 μg) were loaded in all lanes.

a protease-insensitive form of core-glycosylated wt CFTR (see Figure 4). To further characterize this new processing intermediate, cells were metabolically labeled and chased for more extended periods of time. When wt, otherwise untreated transfectants were pulsed for 1 h and subsequently chased for 2.5 h, only the complex-glycosylated form of CFTR was observed, consistent with the fast turnover of the core-glycosylated species (Figure 6A,

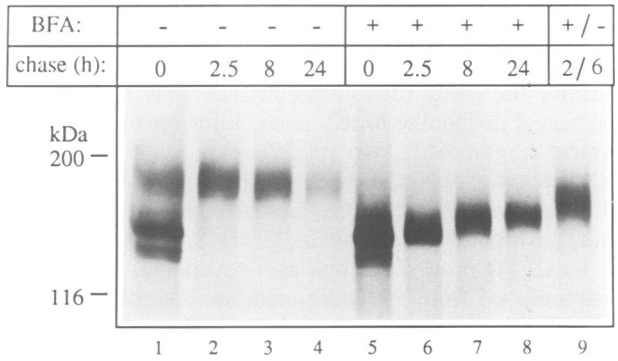

Fig. 6. Comparison of the stability of the complex glycosylated and protease-resistant, core-glycosylated forms of wt CFTR. CHO cells expressing wt CFTR were labeled (1 h) and chased in the absence (lanes 1–4) or presence (lanes 5–8) of BFA (10 μg/ml) for the indicated period. Cell lysates were then prepared and processed as described for Figure 1. In lane 9, the chase medium contained BFA for the first 2 h, but the drug was then removed for the subsequent 6 h. The fluorogram is representative of three experiments.

lanes 1–4). The complex-glycosylated wt CFTR, which turns over much more slowly, was clearly detectable even after 24 h. In three similar experiments, the $t_{\frac{1}{2}}$ of the complex-glycosylated species was estimated to be 16 h. Importantly, the core-glycosylated, protease-resistant form of CFTR that appears in cells treated with BFA was also found to be stable not only in intact (Figure 6A, lanes 5–8), but also in permeabilized cells (Figure 3, lanes 8–10). Unlike the core-glycosylated protein of untreated cells or the large fraction of the protein that is rapidly degraded even after BFA, which have a $t_{\frac{1}{2}} \approx 0.5$ h, the resistant fraction has a much longer $t_{\frac{1}{2}}$ (>24 h, Figure 6A, lanes 5–8). In fact, the resistant core-glycosylated form of BFA-treated cells has a slower turnover than the mature protein of untreated cells. This probably indicates that, when trapped in the ER, the core-glycosylated species is not subject to lysosomal proteolysis, which is thought to contribute to the degradation of the mature CFTR (Yang *et al.*, 1993; Lukacs *et al.*, 1993b). The appearance of a protease-resistant form in the ER of BFA-treated cells suggests that processing of the high-mannose, *N*-linked oligosaccharides into a complex structure is not essential for stabilization of the core-glycosylated CFTR.

The moderate, but progressive decrease in the electrophoretic mobility of the core-glycosylated CFTR observed upon addition of BFA (Figure 6, lanes 5–8) is conceivably due to the modification of carbohydrate chains by Golgi enzymes relocated to the ER by retrograde fusion (Misumi *et al.*, 1986; Lipincott-Schwartz *et al.*, 1990; Sampath *et al.*, 1992). Accordingly, inhibition of retrograde vesicular transport from the Golgi to the ER by disruption of microtubules with nocodazole delayed the appearance of the mobility shift (data not shown).

In full agreement with its stable nature, accumulation of the protease-resistant, core-glycosylated form of wt CFTR was also observed by immunoblot analysis following treatment of transfected CHO cells with BFA for 24 h. A gradual BFA-induced increase in the steady-state content of core-glycosylated wt CFTR was also observed in epithelia expressing the protein either constitutively (T84 cells) or as a result of transfection (PANC-1 cells) (not shown). These findings imply that appearance of the

protease-resistant, core-glycosylated form is not the result of heterologous expression of CFTR in apolar cells. Of note, the steady-state expression level of ΔF508 CFTR was not altered by BFA, in accordance with the failure of the fungal metabolite to reduce the turnover of the mutant protein (Figure 4).

As reported earlier, the effect of BFA on the distribution of Golgi markers in CHO cells was found to be reversible (not illustrated; Klausner *et al.*, 1992). Similarly, the block of wt CFTR processing was also reversible. When BFA was removed from the chase medium, the core-glycosylated CFTR acquired the complex *N*-linked glycosylation pattern over 6 h, as indicated by the mobility shift on SDS–PAGE (Figure 6A, lane 9). This observation suggests that the protease-resistant, core-glycosylated form of wt CFTR that becomes apparent after BFA treatment is a naturally occurring intermediate and not an abnormal, off-pathway by-product. We hypothesize that, under physiological conditions, visualization of this natural folding intermediate is precluded by its immediate release from the ER and subsequent conversion to the mature complex-glycosylated species in the medial Golgi.

Based on these observations we propose that at least two distinct core-glycosylated forms exist along the post-translational folding pathway of CFTR: a newly synthesized, protease-susceptible intermediate that is incompletely folded, which is converted with low efficiency to the protease-resistant and transport-competent form, which is rapidly released from the ER. If this working hypothesis were correct, the amount of core-glycosylated, protease-resistant wt CFTR that accumulates in the ER in the presence of BFA should be equivalent to the amount of complex-glycosylated protein generated over a comparable period in the absence of BFA. To test this hypothesis, we compared the amount of core-glycosylated, protease-resistant wt CFTR with that of fully mature, complex-glycosylated wt CFTR after a 2.5 h chase in the presence or absence respectively of BFA. The length of the chase period was chosen to allow for complete degradation of the protease-susceptible CFTR and for maturation of the complex-glycosylated form (see Figure 2). Quantitative phosphorimage analysis revealed that the amounts of core- and complex-glycosylated wt CFTR were very similar not only in CHO cells, but in BHK cells as well (Figures 4B and 7). This relationship was independent of the duration of the radiolabeling period, confirming the adequacy of the pulse-chase protocol.

It is noteworthy that only ≈15–20% of the pulse-labeled wt CFTR was converted to the protease-resistant, core-glycosylated form in the ER and attained complex glycosylation in the medial Golgi (Figures 4 and 7), in accordance with earlier results (Cheng *et al.*, 1990; Pind *et al.*, 1994; but see Yang *et al.* 1993 for seemingly discrepant results). This low processing efficiency does not seem to correlate with the absolute levels of wt CFTR expression, since similar results were obtained using another clone of CHO cells expressing ~10% as much CFTR (data not shown). Low folding efficiency may instead be the result of heterologous expression in non-epithelial cells. To test this possibility, the precursor–product relationship between the folding intermediate and the complex-glycosylated wt CFTR was also examined in epithelia endogenously expressing CFTR. The results are

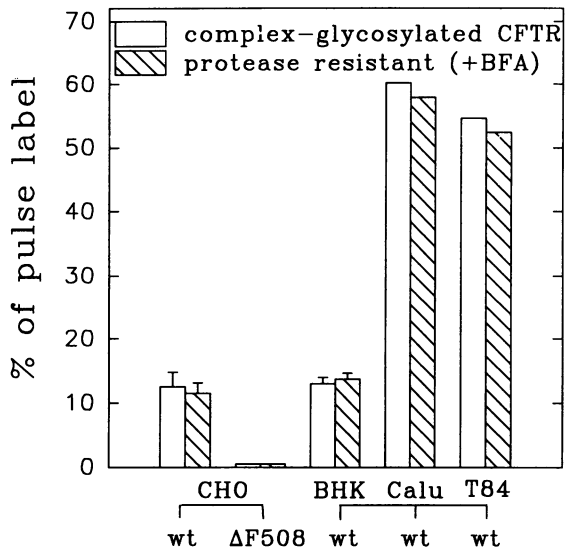

Fig. 7. Relationship between the amounts of core-glycosylated, protease-resistant CFTR and fully mature, complex-glycosylated CFTR in four different cell lines. The indicated cells were pulse-labeled for 15 min and next chased in the presence or absence of BFA (10 μg/ml) for 2.5 h. Cells were then extracted, immunoprecipitated and subjected to SDS–PAGE and quantitative phosphorimaging of wild-type (wt) or mutant (ΔF508) CFTR. The amount of complex-glycosylated CFTR formed (open bars) was determined in untreated cells. Core-glycosylated, protease-resistant CFTR (lined bars) was quantitated in BFA-treated cells. Data are means of 2–4 experiments. In experiments with ≥3 determinations bars represent ± 1 SEM.

summarized in Figure 7. In both T84 and Calu-3 cells the amount of protease-resistant, core-glycosylated CFTR accumulated in the presence of BFA was comparable with the amount of fully mature, complex-glycosylated CFTR. While the maturation efficiency of wt CFTR in these epithelia was higher than in transfected fibroblasts (≈55 versus 15%), resembling findings in transfected C127 and LLCPK epithelia (Marshall *et al.*, 1994), a significant fraction of the protein was nevertheless degraded prior to maturation. These data strongly support the hypothesis that conformational maturation of the core-glycosylated wt CFTR is a comparatively inefficient step that occurs in the ER and is a prerequisite for stabilization and export of the protein to the Golgi.

In an attempt to inhibit degradation of core-glycosylated CFTR and ΔF508 CFTR and promote more efficient conformational maturation, we tested several agents known to block proteolysis in pre-Golgi compartments (Klausner and Sitia, 1990; Bonifacino and Lipincott-Schwartz, 1991). Among the inhibitors tested (see Table I for details) only ALLN had a moderate effect on the degradation rate of core-glycosylated CFTR and ΔF508 CFTR. Moreover, degradation of both the wt and mutant proteins persisted after inhibition of protein synthesis with cycloheximide, implying that short-lived proteins do not participate in the breakdown of CFTR (Table I).

ATP is required for conformational maturation of core-glycosylated wt CFTR in the ER

It has been reported that cytosolic ATP is required for the proper folding and assembly of certain proteins (Doms *et al.*, 1987; Levy *et al.*, 1991; Braakman *et al.*, 1992). Since the conformational transition of wt CFTR can be

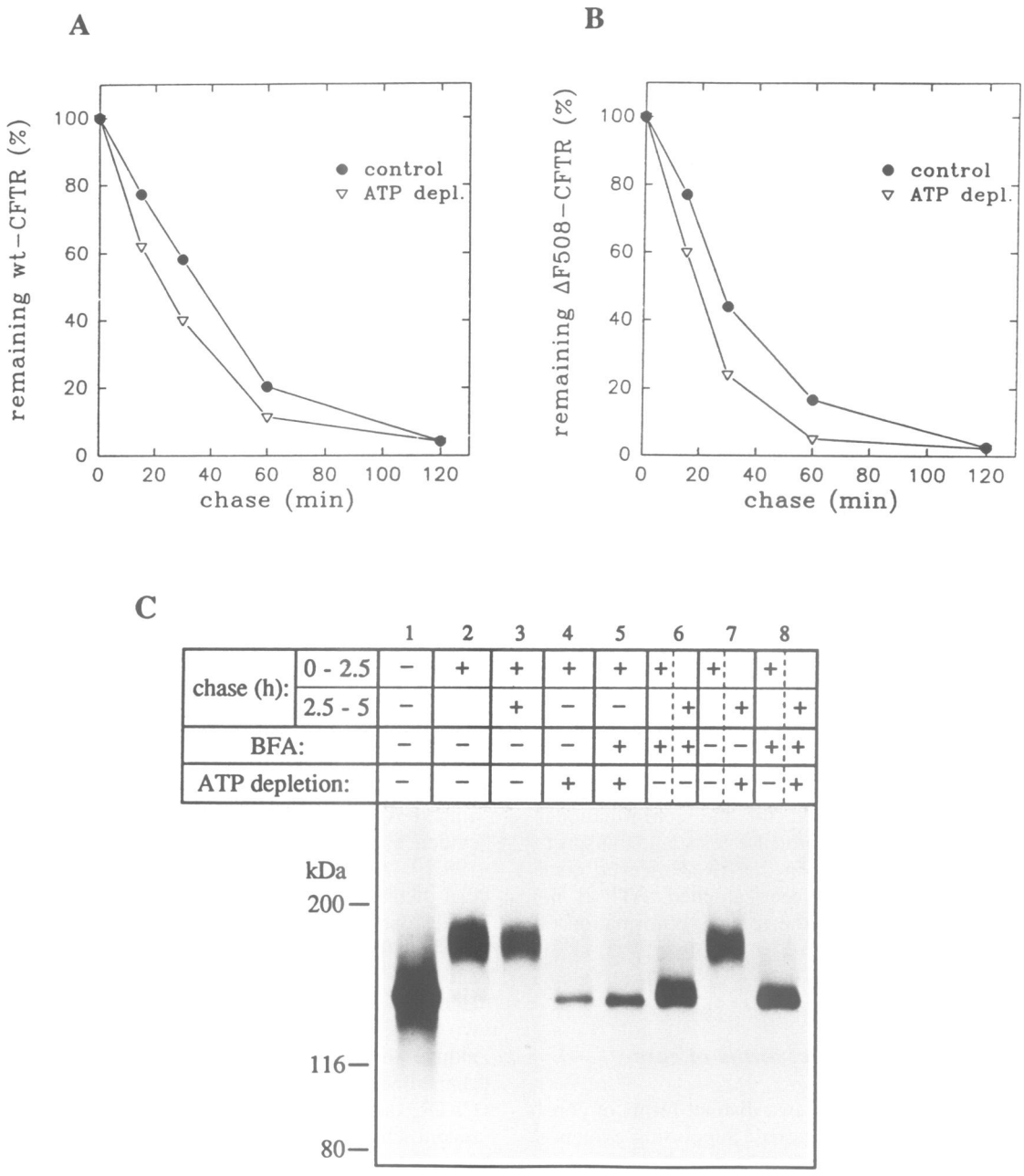

Fig. 8. Effect of cytosolic ATP depletion on the stability of wt CFTR and ΔF508 CFTR. The turnover of core-glycosylated wt and ΔF508 CFTR was measured by pulse-chasing, immunoprecipitation and phosphorimaging as described in the legend to Figure 2. After pulse-labeling, some samples were subjected to ATP depletion by addition of metabolic inhibitors (10 mM deoxy-D-glucose and 4 μg/ml antimycin) plus a protonophore (10 μM CCCP) in the chase medium. (**A**) The amount of residual core-glycosylated wt CFTR was studied as a function of time in untreated (solid circles) and ATP-depleted cells (open triangles). (**B**) Amount of residual core-glycosylated ΔF508 CFTR in untreated (solid circles) and ATP-depleted cells (open triangles). (**C**) CHO cells expressing wt CFTR were labeled for 20 min and chased for either 2.5 or 5 h, as specified. Where indicated, BFA (10 μg/ml) was added during the first and/or second chase period. ATP depletion, accomplished as described above, was performed where specified. Note that some samples (lanes 6–8) were chased in media of different composition during the first and second 2.5 h periods.

dissociated from its translocation to the Golgi by using BFA, we tested whether ATP is required for the conformational maturation step. Pulse-chase experiments were performed and, where indicated, the ATP content of CHO cells was reduced by ⩾96% in 5 min, using a combination of antimycin, CCCP and deoxy-D-glucose. The rate of disappearance of core-glycosylated wt CFTR was modestly accelerated by depletion of ATP (Figure 8A). Similar results were obtained for ΔF508 CFTR (Figure 8B). ATP-independent protein degradation in a pre-Golgi compartment has been reported in other systems (Stafford and Bonifacino, 1991; Wikstrom and Lodish, 1992; Young *et al.*, 1993).

As anticipated, depletion of ATP completely abrogated the appearance of complex-glycosylated wt CFTR (cf. lanes 2 and 4 in Figure 8C), consistent with the energy dependence of ER-to-Golgi traffic (Jamieson and Palade, 1968). Together with the findings in Figure 8A and B, these observations imply that degradation of newly synthesized, core-glycosylated wt CFTR (and ΔF508

CFTR) does not depend on vesicular transport, in agreement with the conclusion reached above using BFA and permeabilized cells.

It is important that not only did degradation of CFTR persist in ATP-depleted cells, but that formation of the protease-resistant, core-glycosylated form was substantially inhibited. This is apparent when comparing the amount of core-glycosylated species remaining in BFA-treated cells with or without ATP depletion (cf. lanes 5 and 6 in Figure 8C). Note that the amount of residual protein is much greater in the presence of ATP, even after a 5 h chase compared with the shorter 2.5 h chase used for ATP-depleted cells. This observation suggests that the conformational transition that renders wt CFTR protease-resistant requires metabolic energy. In the absence of the nucleotide, the protein fails to accumulate even in the presence of BFA. The small amount of protease-resistant, core-glycosylated wt CFTR detectable in the presence of metabolic inhibitors (lanes 4 and 5 in Figure 8C) was conceivably formed during metabolic labeling of the cells, prior to ATP depletion.

Interestingly, metabolic energy is not required to preserve the stability of wt CFTR after it has undergone the conformational transition. Only marginal degradation was observed when pulse-labeled wt CFTR was allowed to attain the protease-resistant conformation prior to depletion of ATP. This observation applies both to the complex-glycosylated species (cf. lanes 3 and 7, Figure 8C) and to the core-glycosylated form detected in the ER of BFA-treated samples (cf. lanes 6 and 8). Together, these findings indicate that ATP is required for the conformational transition that generates the protease-resistant, transport-competent form of wt CFTR in the ER. However, once the stable conformation has been attained, ATP is no longer necessary to maintain the folded conformation of wt CFTR.

Discussion

Identification of two distinct forms of core-glycosylated wt CFTR

In this report, the existence of two distinct forms of core-glycosylated wt CFTR is suggested. Supporting evidence is based mainly on the biphasic nature of the disappearance of pulse-chased wt CFTR: one fraction of the protein is rapidly degraded ($t_{\frac{1}{2}} \approx 35$ min), while the remainder acquires protease resistance and the ability to reach the Golgi, where it undergoes further modifications of the *N*-linked oligosaccharides. The latter has a considerably longer $t_{\frac{1}{2}}$ ($\approx$16 h). Importantly, attainment of protease resistance requires neither complex glycosylation nor departure from the ER, since a stable form of core-glycosylated CFTR accumulates in the ER of BFA-treated cells.

We propose that the protease-sensitive form of core-glycosylated wt CFTR is the natural precursor of the protease-resistant species. The conversion process appears to be rather inefficient, as only a fraction of the synthesized protein (20–50%) reaches the protease-resistant stage. In contrast, transit of the latter to the Golgi and complex glycosylation are highly efficient events, as indicated by the similar magnitude of the pools of protease-resistant, core-glycosylated wt CFTR (detected after BFA) and of the fully mature protein (e.g. Figure 7). This quantitative relationship between the two stable forms of CFTR was valid not only in transfected CHO and BHK cells, but also in the epithelial lines T84 and Calu-3, which express wt CFTR endogenously. Thus, while not distinguishable by their mobility in SDS–PAGE, two distinct species of core-glycosylated wt CFTR appear to exist, differing most likely in their tertiary conformation and/or in the state of association with other components of the ER.

Post-translational maturation of wt CFTR is dependent on metabolic energy

Conversion of the core-glycosylated wt CFTR to the mature species is seemingly dependent on the availability of ATP. This is due in part to the abrogation of vesicular traffic between the ER and Golgi. However, experiments using BFA demonstrated that, in addition, ATP is required for the conformational transition that leads to the acquisition of protease resistance by the core-glycosylated intermediate (Figure 8). A requirement for ATP for the correct folding of other proteins in the ER had been described earlier (Doms *et al.*, 1987; Levy *et al.*, 1991; Braakman *et al.*, 1992).

The precise role of ATP in the conformational maturation of CFTR is presently unclear, but several possibilities can be envisaged. First, binding of ATP to one or both nucleotide binding domains (NBD) of the protein may be required to facilitate the structural conversion. Consistent with this notion, the inability of the ΔF508 mutant to undergo maturation may be related to the moderately altered ATP binding by the first NBD, as suggested by evidence obtained on synthetic peptides (Thomas *et al.*, 1992b). Alternatively, ATP may bind to other proteins that dictate the fate of CFTR. Such proteins may include molecular chaperones, like the heat shock proteins (hsp70 and hsp60), known to possess nucleotide binding domains and ATPase activity (Gething and Sambrook, 1992; Hendrick and Hartl, 1993). Binding of the chaperones to wt CFTR in the presence of ATP may be necessary to induce the conformational maturation. Conversely, the chaperones may sequester the inappropriately folded CFTR, targeting it for degradation. This notion is consistent with the finding that both the wild-type and mutant forms of CFTR were found to form complexes with hsp70 (Yang *et al.*, 1993) and calnexin (Pind *et al.*, 1994). In the latter model, ATP may be necessary to release the misfolded core-glycosylated CFTR from its complex with the chaperone, enabling it to assume the appropriate conformation. Clearly, dissociation from the chaperone(s) and folding may be coupled events, conceivably requiring the hydrolysis of ATP, as has been demonstrated (Pelhalm, 1989; Craig, 1993; Hendrick and Hartl, 1993). Finally, ATP might be necessary to phosphorylate one of the multiple consensus phosphorylation sites on CFTR, a step that may be required for maturation.

The ΔF508 mutation blocks the conformational transition between the two core-glycosylated forms of CFTR

As described earlier (Cheng *et al.*,1990; Yang *et al.*,1993), ΔF508 CFTR is unable to attain the complex-glycosylated form in the medial Golgi and to reach the plasma membrane, being instead degraded intracellularly. It is remark-

able that the rate of degradation of ΔF508 CFTR is virtually identical to the rate of disappearance of the protease-susceptible component of core-glycosylated wt CFTR. This observation suggests that the wild-type and mutant proteins share an early conformation which is equally susceptible to proteolysis. It follows that the inability of ΔF508 CFTR to mature is due to its failure to attain the protease-resistant, core-glycosylated stage.

This interpretation differs subtly yet significantly from the current model, wherein the wild-type protein is co-translationally folded into the appropriate configuration, while the mutant never attains this stage (Cheng *et al.*, 1990; Thomas *et al.*, 1992a; Yang *et al.*, 1993). Not only is the wild-type protein not immediately folded, but the folding process is comparatively slow and inefficient, possibly owing to the complex, multi-domain structure of CFTR. A corollary of the present hypothesis is that more functional wt CFTR would be able to reach the plasma membrane if degradation in the ER is prevented and/or if the folding process is accelerated.

Where does degradation of incompletely folded wt CFTR and ΔF508 CFTR occur?

Proteolysis of CFTR in a compartment situated at an early stage in the biosynthetic pathway is not unique. Degradation of newly synthesized, misfolded or incompletely assembled proteins in a pre-Golgi compartment has been described previously (Hurtley and Helenius, 1989; Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991). However, the precise identity of this compartment remains to be defined (Klausner and Sitia, 1990; Sitia and Meldolesi, 1992). In this study we provide further information regarding the site of degradation of wt and ΔF508 CFTR. First, degradation was insensitive to lysosomotropic agents and to lysosomal protease inhibitors. Second, proteolysis was unaffected when vesicular transport between the ER and the Golgi was disrupted by either permeabilization, BFA or ATP depletion (Figures 3, 4 and 8). Third, the degradation process was initiated without a significant time lag and was substantially inhibited at reduced temperatures, characteristics that are typical of proteolysis in pre-Golgi compartments (Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991). Finally, proteolytic intermediates of ΔF508 CFTR degradation were detectable in isolated vesicles enriched in sulfatase C, an ER marker (Figure 5). These cumulative results point to the ER as the primary site of CFTR degradation. While degradation of ΔF508 CFTR may not occur exclusively in this compartment, our findings suggest that at least a significant portion of the mutant protein, and by inference also the rapidly disappearing fraction of wt CFTR, are broken down in the ER.

In summary, we have described two distinct forms of core-glycosylated wt CFTR: one that is susceptible to proteolysis and another that is resistant. These are interpreted to be sequential stages in the maturation of the protein. The conformational transition that enables the maturation of wt CFTR was found to be an ATP-dependent process occurring in the ER. Finally, it is suggested that the inability of ΔF508 CFTR to undergo such a transition renders it susceptible to complete and rapid degradation in the ER.

Materials and methods

Cell lines

Stable transfectants of Chinese hamster ovary-K1 (CHO) cells expressing human wt CFTR or ΔF508 CFTR (see Tabcharani *et al.*, 1992; Chang *et al.*, 1993 for details) were maintained in αMEM containing 7% fetal calf serum, antibiotics and 200 μM methotrexate (Tabcharani *et al.*, 1992; Lukacs *et al.*, 1993a). Baby hamster kidney (BHK) cell lines were generated similarly. Clones were selected and propagated in a 1:1 mixture of F12 and DMEM media containing 10% serum and 500 μM methotrexate. The human colon carcinoma line T84 was grown as described (Sood *et al.*, 1992). The human lung adenocarcinoma cell line Calu-3 of submucosal gland origin (ATCC HTB 55; Shen *et al.*; 1994) was maintained in DMEM supplemented with 10% serum. CFTR-deficient pancreatic ductal cells (PANC-1) were stably transfected with the pMT/EP expression vector (kindly provided by Dr Joseph Ilan; Trojan *et al.*, 1993) containing the full-length wt CFTR or ΔF508 CFTR cDNA. pMT/EB-wt CFTR and pMT/EP-ΔF508 CFTR were constructed by subcloning the appropriate *Not*I–*Xho*I fragment of pNUT-wt CFTR or pNUT-ΔF508 CFTR. Clones were isolated, screened by immunoblotting with anti-CFTR antibodies (Kartner *et al.*, 1992) and propagated in DMEM supplemented with 10% serum and 500 μg/ml hygromycin.

Metabolic labeling and immunoprecipitation

Metabolic labeling and immunoprecipitation were carried out essentially as described earlier (Pind *et al.*, 1994). After incubation in methionine- and cysteine-free αMEM for 30 min at 37°C, transfected and epithelial cells were pulsed in the same medium containing 140 μCi/ml (CHO and BHK cells) or 300 μCi/ml (T84 and Calu-3) [^{35}S]methionine and [^{35}S]cysteine (>1000 Ci/mmol; Amersham Corp.) for the indicated time at 37°C. For chasing, the labeling medium was replaced with complete αMEM supplemented with 7% serum and 0.75 mM methionine.

Metabolically labeled CFTR was isolated by immunoprecipitation. After washing the monolayers with ice-cold Hank's balanced salt solution (HBSS), cells were solubilized in 1 ml RIPA buffer (150 mM NaCl, 20 mM Tris–HCl, 1% Triton X-100, 0.1% SDS and 0.5% deoxycholate, pH 8.0) containing 10 μg/ml each of leupeptin, pepstatin, chymostatin and antipain plus 10 mM iodoacetamide for 30 min at 4°C. Insoluble material was sedimented by centrifugation at 15 000×*g* for 15 min at 4°C. The supernatant was incubated for 2 h at 4°C with a mixture of two monoclonal anti-CFTR antibodies (M3A7 and L12B4, 1.2 μg/ml each; Kartner *et al.*, 1992) and immune complexes were precipitated with protein G–agarose beads (Sigma) for 1 h at 4°C, followed by eight washes with RIPA buffer. Immunoprecipitated proteins were eluted and denatured with 2× concentrated Laemmli sample buffer and analyzed by electrophoresis.

Electrophoresis, immunoblotting and phosphorimage analysis

Protein samples were analyzed after separation by SDS–PAGE on 6 or 7.5% gels (Laemmli, 1970). Immunoblotting was performed as described earlier (Lukacs *et al.*, 1993a) using the M3A7 anti-CFTR antibody. For fluorography gels were fixed in 10% acetic acid and 40% ethanol, soaked for 30 min in Amplify (Amersham) and dried. Labelled proteins were visualized by exposing the gels to Kodak X-OMAT AR or Dupont NEF 496 film at −70°C for 1–3 days. The radioactivity associated with CFTR was quantified using a PhosphorImager (PDI) with ImageQuant software.

Subcelluar fractionation

Isolation of ER-, Golgi- and plasma membrane-enriched fractions from CHO cells expressing ΔF508 CFTR was performed by a modification of the protocol of Bole *et al.* (1986). The cells (≈5×10^8) were grown in suspension, harvested and washed in ice-cold HBSS by centrifugation. After an additional wash in sucrose medium (0.25 M sucrose, 5 mM HEPES, 1 mM EDTA, pH 7.2), cells were homogenized by nitrogen cavitation (350 p.s.i., 15 min at 4°C) in sucrose medium supplemented with a protease inhibitor cocktail (5 μg/ml each of chymostatin, pepstatin, leupeptin and antipain). The post-nuclear supernatant was fractionated by sedimentation on a discontinuous sucrose gradient (Bole *et al.*, 1986). Fractions (1.5 ml) were collected from the bottom of the tubes with an Autodensi-flow fraction collector (Buchler). The activities of alkaline phosphatase, sulfatase C and α-mannosidase II, specific marker enzymes of plasma membrane, ER and Golgi complex respectively, were determined in each fraction (Storrie and Madden, 1990).

Other methods

To produce 'semi-intact' cells, CHO transfectants expressing wt CFTR or ΔF508 CFTR were mechanically perforated after hypotonic swelling according to the protocol of Beckers *et al.* (1987). Permeabilization efficiency was more than 95%, as evaluated by Trypan blue staining. The ATP content of tricholoroacetic acid extracts of control and metabolically depleted cells was determined using the luciferin−luciferase Calbiochem kit. Protein content was measured with the BCA kit (Pierce) using bovine serum albumin as the standard.

Acknowledgements

We would like to thank Drs K.Moremann and M.Farquhar for making available the polyclonal anti-mannosidase II antibody and Dr Joseph Ilan for providing the pMT/EP plasmid. We thank Drs S.Pind and C.Hammond for helpful advice. This work was supported by the Canadian and US Cystic Fibrosis Foundations and by the Medical Research Council of Canada. G.L.L. was supported by a Postdoctoral Fellowship of the Canadian Cystic Fibrosis Foundation until June 1994. Presently he is Assistant Professor and cross-appointed to the Department of Surgery of the University of Toronto. A.M.was partially supported by a Postdoctoral Fellowship from the Swedish Institute and the Canadian Cystic Fibrosis Foundation. S.G. is an International Scholar of the Howard Hughes Medical Institute and is cross-appointed to the Department of Biochemistry of the University of Toronto.

References

Amitay,R., Shachar,I., Rabinovich,E., Haimovich,J. and Bar-Nun,S. (1992) *J. Biol. Chem.*, **267**, 20694–20700.

Anderson,M.P., Berger,H.A., Rich,D.P., Gregory,R.J., Smith,A.E. and Welsh,M.J. (1991) *Cell*, **67**, 775–784.

Bear,C.E., Li,C., Kartner,N., Bridges,R.J., Jensen,T.J., Ramjeesingh,M. and Riordan,J. R. (1992) *Cell*, **68**, 809–818.

Beckers,C.J.M., Keller,D.S. and Balch,W.E. (1987) *Cell*, **50**, 523–531.

Biwersi,J. and Verkman,A.S. (1994) *Am. J. Physiol. Cell*, **266**, C149-C155.

Bole,D.G., Hendershot,L.M. and Kearney,J.F (1986) *J. Cell Biol.*, **102**, 1558–1566.

Bonifacino,J.S. and Lippincott-Schwartz,J. (1991) *Curr. Opin. Cell Biol.*, **3**, 592–600.

Braakman,I., Helenius,J., and Helenius,A. (1992) *Nature*, **356**, 260–262.

Chang,X.-B., Tabcharani,J.A., Hou,Y.-X., Jensen,T., Karnter,N., Alon,N., Hanrahan,J.H. and Riordan,J.R. (1993) *J. Biol. Chem.*, **268**, 11304–11311.

Cheng,S.H., Gregory,R.J., Marshall,J., Paul,S., Souza,D., White,G.A., O'Riordan,C.R. and Smith,A.E. (1990) *Cell*, **63**, 827–834.

Craig,E.A. (1993) *Science*, **260**, 1902–1904.

Denning,G.M., Anderson,M.P., Amara,J.F., Marshall,J., Smith,A.E. and Welsh,M.J. (1992a) *Nature*, **358**, 761–764.

Denning,G.M., Ostedgaard,L.S. and Welsh,M.J. (1992b) *J. Cell Biol.*, **118**, 551–559.

Doms,R.W., Keller,D.S., Helenius,A. and Balch,W.E. (1987) *J. Cell Biol.*, **105**, 1957–1969.

Drumm,M.L., Wilkinson,D.J., Smit,L.S., Worell,R.T., Strong,T.V., Frizzell,R.A., Dawson,D.C. and Collins,F.S. (1991) *Science*, **254**, 1797–1799.

Engelhardt,J.F., Yankaskas,J.R., Ernst,S.A., Yang,Y., Marino,C.R., Boucher,R.C., Cohn,J.A. and Wilson,J.M. (1992) *Nature Genet.*, **2**, 240–248.

Gething,M.J. and Sambrook,J. (1992) *Nature*, **355**, 33–45.

Hammond,C. and Helenius,A. (1994) *J. Cell Biol.*, **126**, 41–52.

Hendrick,J.P. and Hartl,F.-U. (1993) *Annu. Rev. Biochem.*, **62**, 349–384.

Holtzman,E. (1989) *Lysosome*. Plenum Press, New York, NY.

Hsu,V.W., Yuan,L.C., Nuchtern,J.G., Lippincott-Schwartz,J., Hammerling, G.J. and Klausner,R.D. (1991) *Nature*, **352**, 441–444.

Hurtley,S.M. and Helenius,A. (1989) *Annu. Rev. Cell Biol.*, **5**, 277–307.

Jamieson,J.D. and Palade,G.E. (1968) *J. Cell Biol.*, **39**, 589–603.

Kartner,N., Augustinas,O., Jensen,T., Naismith,A.L. and Riordan,J. (1992) *Nature Genet.*, **1**, 321–327.

Kerem,B.-S., Rommens,J.M., Buchanan,J.A., Markiewicz,D., Cox,T.K., Chakravarti,A., Buchwald,M. and Tsui,L.-C. (1989) *Science*, **245**, 1073–1080.

Klausner,R.D. and Sitia,R. (1990) *Cell*, **62**, 611–614.

Klausner,R.D., Donaldson,J.G. and Lipincott,S.J. (1992) *J. Cell Biol.*, **116**, 1071–1080.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Levy,F., Gabathuler,R., Larsson,R. and Kvist,S. (1991) *Cell*, **67**, 265–274.

Li,C., Ramjeesingh,M., Reyes,E., Jensen,T., Chang,X.-B., Rommens,J.M. and Bear,C.E. (1993) *Nature Genet.*, **3**, 311–316.

Lippincott-Schwartz,J., Donaldson,J.G., Schweizer,A., Berger,E.G., Hauri,H.-P., Yuan,L.C. and Klausner,R.D. (1990) *Cell*, **60**, 821–836.

Lukacs,G.L., Chang,X.-B., Kartner,N., Rotstein,O.D., Riordan,J.R. and Grinstein,S. (1992) *J. Biol. Chem.*, 267, 14568–14572

Lukacs,G.L., Chang,X., Bear,C., Kartner,N., Mohamed,A., Riordan,J.R. and Grinstein,S. (1993a) *J. Biol. Chem.*, **268**, 21592–21598.

Lukacs, G.L., Gaisinsky, I., Trudel, S., Kartner, N., Chang, X.-B., Riordan, J. R., and Grinstein, S. (1993b) *Biochem. Cell Biol.*, 71, A24

Marshall,J., Fang,S., Ostedgaard,L.S., O'Riordan,C.R., Ferrara,D., Amara,J.F., Hoppe,H., Welsh,M.J., Smith,A.E. and Cheng,S.H. (1994) *J. Biol. Chem.*, **269**, 2987–2995.

Misumi,Y., Miki,A., Takatsuki,G., Tamura,G. and Ikehara,Y. (1986) *J. Biol. Chem.*, **261**, 11398–11403.

Moremen,K.W. and Robbins,P.W. (1991) *J. Cell Biol.*, **115**, 1521–1534.

Pind,S., Riordan,J.R. and Williams,D.B (1994) *J. Biol. Chem.*, **269**, 12748–12788.

Quinton,P.M. (1990) *FASEB J.*, **4**, 2709–2717.

Pelham,H.R.B. (1989) *Annu. Rev. Cell Biol.*, **5**, 1–23.

Riordan,J.R. (1993) *Annu. Rev. Physiol.*, **55**, 609–630.

Riordan,J.R., Rommens,J.M., Kerem,B.-S., Alon,N., Rozmahel,R., Grzelczak,Z., Zielenski,J., Lok,S., Plavsic,N., Chou,J.-L., Drumm,M.-L., Iannuzzi,M.C., Collins,F.S. and Tsui,L.-C. (1989) *Science*, **245**, 1066–1073.

Rommens,J.M., Iannuzzi,M.C., Kerem,B.-S., Drumm,M.L., Melmer,G., Dean,M., Rozmahel,R., Cole,J.L., Kennedy,D., Hidaka,N., Zsiga,M., Buchwald,M., Riordan,J.R., Tsui,L.-C. and Collins,F.S. (1989) *Science*, **245**, 1059–1065.

Sampath,D., Varki,A. and Freeze,H.H. (1992) *J. Biol. Chem.*, **267**, 4440–4455.

Sferra,T.J. and Collins,F.S. (1993) *Annu. Rev. Med.*, **44**, 133–144.

Shen,B.-Q., Finkbemer,W.E., Wine,J.J., Mrony,R.J. and Widdicombe,J.H. (1994) *Am. J. Physiol. Lung*, **266**, 493–501.

Sitia,R. and Meldolesi,J. (1992) *Mol. Biol. Cell.*, **259**, 1901–1904.

Sood,R., Bear,C., Auerbach,W., Reyes,E., Jensen,T., Kartner,T., Riordan,J.R. and Buchwald,M. (1992) *EMBO J.*, **11**, 2487–2494.

Stafford,F.J. and Bonifacino,J.S. (1991) *J. Cell Biol.*, **115**, 1225–1236.

Storrie,B. and Madden,E.A. (1990) *Methods Enzymol.*, **182**, 203–224.

Tabcharani,J.A., Chang,X.-B., Kartner,N., Hanrahan,J.R. and Riordan,J.R. (1991) *Nature*, **352**, 628–632.

Teem,J.B., Berger,H.A., Ostedgaard,L.S., Rich,D.P., Tsui,L.-C. and Welsh,M.J. (1993) *Cell*, **73**, 335–346.

Thomas,P.J., Ko,Y.H. and Pedersen,P.L. (1992a) *FEBS Lett.*, **312**, 7–9.

Thomas,P.J., Shenbagamurti,P., Sondek,J., Hullihen,J. and Pedersen,P.L. (1992b) *J. Biol. Chem.*, **267**, 5727–5730.

Trojan,J., Blossey,B.K., Johnson,T.R., Rudin,S.D., Tykocynski,M.L., Ilan,Ju. and Ilan,J. (1993) *Proc. Natl Acad. Sci. USA*, **89**, 4874–4878.

Tsui,L.-C. (1992) *Trends Genet.*, **8**, 392–398.

Welsh,M.J. and Smith,A.E. (1993) *Cell*, **73**, 1251–1254.

Wikstrom,L. and Lodish,H.F. (1992) *J. Biol. Chem.*, **267**, 5–8.

Yang,Y., Janich,S., Cohn,J.A. and Wilson,J.M. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 9480–9484.

Young,J., Kane,L.P., Exley,M. and Wileman,T. (1993) *J. Biol. Chem.* **268**, 19810–19818.

Received on August 24, 1994; revised on October 11, 1994