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Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome

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Maturation of wild-type CFTR nascent chains at the endoplasmic reticulum (ER) occurs inefficiently; many disease-associated mutant forms do not mature but instead are eliminated by proteolysis involving the cytosolic proteasome. Although calnexin binds nascent CFTR via its oligosaccharide chains in the ER lumen and Hsp70 binds CFTR cytoplasmic domains, perturbation of these interactions alone is without major influence on maturation or degradation. We show that the ansamysin drugs, geldanamycin and herbimycin A, which inhibit the assembly of some signaling molecules by binding to specific sites on Hsp90 in the cytosol or Grp94 in the ER lumen, block the maturation of nascent CFTR and accelerate its degradation. The immature CFTR molecule was detected in association with Hsp90 but not with Grp94, and geldanamycin prevented the Hsp90 association. The drug-enhanced degradation was decreased by lactacystin and other proteasome inhibitors. Therefore, consistent with other examples of countervailing effects of Hsp90 and the proteasome, it would seem that this chaperone may normally contribute to CFTR folding and, when this function is interfered with by an ansamycin, there is a further shift to proteolytic degradation. This is the first direct evidence of a role for Hsp90 in the maturation of a newly synthesized integral membrane protein by interaction with its cytoplasmic domains on the ER surface.

Keywords: ansamycin/CFTR/ER degradation/Hsp90/proteasome

Introduction

Most patients with cystic fibrosis (CF) have the ΔF508 mutation on at least one CFTR gene allele (Collins, 1992). As a consequence, the protein product of that allele, while potentially competent to perform as a regulated chloride channel at the cell surface, is biosynthetically misprocessed and mislocalized (Cheng *et al.*, 1991). Although completed core-glycosylated nascent chains are formed in the rough endoplasmic reticulum (ER), they are unable to mature conformationally (Lukacs *et al.*, 1994) and hence are precluded from exiting from the ER. It is generally believed, and there is some supporting experimental evidence (Qu and Thomas, 1996; Qu *et al.*, 1997), that misfolding occurs due to the absence of Phe508. The aberrant molecule is degraded at the ER at least in part by the cytosolic proteasome (Jensen *et al.*, 1995; Ward *et al.*, 1995). This initially was viewed as a novel means of proteolysis of newly synthesized polypeptides in the early secretory pathway (Sommer and Jentsch, 1993), but there is now extensive evidence that this degradative route is an integral part of the quality control system which scrutinizes nascent secretory and membrane proteins, serving to dispose of those which have not acquired a native conformation (Sommer and Wolf, 1997). Particularly intriguing have been several indications that both soluble secretory proteins and integral membrane proteins may be translocated from within the ER to its exterior (cytoplasmic face) to be made accessible to the proteasome (Biederer *et al.*, 1997). However, in the case of CFTR where the greatest part of its mass is cytoplasmically exposed, such retrotranslocation may not need to be involved.

Nevertheless, it is clear that nascent CFTR is recognized by components of the overall quality control apparatus other than just the proteasome. Indeed, interactions with the ER membrane chaperone, calnexin (Pind *et al.*, 1994), and cytosolic Hsp70 (Yang *et al.*, 1993) have been demonstrated. Both of these molecular chaperones have well-documented roles in the promotion of protein folding (Chen *et al.*, 1995; Hebert *et al.*, 1995; Frydman and Hohfeld, 1997; Johnson and Craig, 1997). Additionally, Hsp70 family members and their cofactors may, after multiple rounds of association and dissociation, direct substrates which remain incompletely folded to the proteasome for degradation (Sherman and Goldberg, 1996). In contrast to the requirement for calnexin association for ER degradation of mutant α_1-antitrypsin (Qu *et al.*, 1996), prevention of calnexin binding to CFTR either by mutagenesis of its *N*-glycosylation sites or castanospermine treatment of cells is without major impact on wild-type CFTR processing (unpublished observations).

Since CFTR's topological disposition makes it available for interactions with additional molecular chaperones on either side of the ER membrane, we have now attempted to identify others which bind nascent CFTR. Special attention was paid to members of the Hsp90 family since they have been found to play a major role both in the prevention of protein aggregation and the facilitation of protein folding or degradation (Smith *et al.*, 1995; Buchner, 1996; Schneider *et al.*, 1996; Dittmar and Pratt, 1997; Grenert *et al.*, 1997; Pratt and Toft, 1997; Scheibel *et al.*, 1998). We have found that cytoplasmic Hsp90 binds to both wild-type and ΔF508 nascent CFTR while Grp94 in the ER lumen does not. The Hsp90 association is disrupted by benzoquinone ansamycin drugs, and this results in a strong acceleration of proteasomal degradation. These

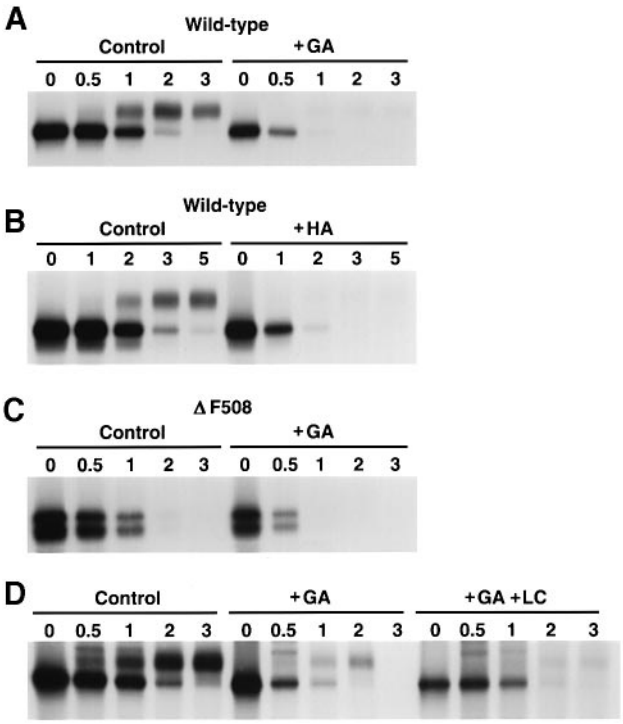

Fig. 1. Effects of ansamysin benzoquinones on the biosynthetic processing of CFTR. CHO or BHK cells stably expressing wild-type or ΔF508 CFTR grown in 6 cm culture dishes were exposed to the drugs for 90 min prior to pre-incubation in methionine-free medium for a further 30 min also in their presence. The cells were then pulse-labeled with [^{35}S]methionine (100 μCi/ml) for 20 min and chased with methionine-containing medium for the times indicated in hours. At these times, the cells were solubilized with the NP-40 lysis buffer and immunoprecipitated with the monoclonal antibody M3A7 [2 μl of ascites fluid (Kartner *et al.*, 1991)] during an overnight incubation at 4°C with gentle rocking, then 25 μl of packed protein G–agarose beads were added and incubation continued for 3 h. Beads were washed four times with excess lysis buffer and eluted with gel loading buffer. Following electrophoresis (7% acrylamide), the gels were dried and exposed to X-ray film to yield the fluorograms shown. (**A**) Geldanamycin (0.1 μg/ml) causes a marked acceleration of disappearance of core-glycosylated wild-type CFTR (lower band) and prevents formation of its maturation product with complex oligosaccharide chains (upper band) in BHK cells. (**B**) Herbimycin A (3 μg/ml) has essentially the same effect on the processing of wild-type CFTR in CHO cells. (**C**) Geldanamycin causes a similar increase in the rate of turnover of the immature core-glycosylated form of ΔF508 CFTR in CHO cells [the lower band of the doublet is the product of translation from an alternate initiation site (S.Pind, A.Mohamed, X.-B.Chang, Y.-X.Hou, D.B.Williams and J.R.Riordan, unpublished)]. (**D**) The proteasome inhibitor lactacystin (Fenteany *et al.*, 1995) delays the enhanced turnover of core-glycosylated wild-type CFTR caused by geldanamycin but does not restore maturation.

findings are consistent with the apparently antagonistic actions of Hsp90 and the proteasome in the turnover of other proteins (Tsubuki *et al.*, 1994; Wagner and Margolis, 1995) and provide the first example of the involvement of this abundant cytosolic chaperone in the biosynthetic processing of nascent integral membrane proteins at the ER.

Results

Ansamycins inhibit CFTR maturation and accelerate degradation

Figure 1 illustrates the results of pulse–chase experiments in which the turnover of the smaller, core-glycosylated precursor form of CFTR and the appearance of its larger, complex-glycosylated product is observed. In agreement with earlier studies in which wild-type and ΔF508 CFTR were expressed in a number of different cell types, the smaller immature band disappears with a half-time of ~30 min, a value which is not significantly different for the two genotypes. In the case of the wild-type, ~25–30% of the precursor is accounted for by the appearance of mature product (Figure 1A and B). No mature product is generated by ΔF508 CFTR (Figure 1C). On treatment of cells with the benzoquinone ansamycin drugs, geldanamycin (Figure 1A) or herbimycin A (Figure 1B), the turnover of the core-glycosylated precursor band is greatly accelerated and no mature product is formed. Hence, these drugs appear to arrest the normal biosynthetic processing of CFTR completely. The rate of disappearance of the immature ΔF508 CFTR form is similarly increased (Figure 1C). The large proportion (70–75%) of wild-type immature band which disappears in the absence of the drugs is proteolysed (Pind *et al.*, 1994) at least in part by the cytoplasmic proteasome (Jensen *et al.*, 1995; Ward *et al.*, 1995). To determine whether the proteasome is involved in the elevated rate of degradation caused by the ansamycins, the effect of the specific proteasome inhibitor, lactacystin, was examined (Figure 1D). Lactacystin slowed the enhanced rate of disappearance appreciably but failed to restore maturation. Therefore, it seems that geldanamycin does increase the susceptibility of nascent CFTR to the proteasome but, as in the absence of ansamycin treatment, inhibition of the proteasome does not enable maturation of that population of nascent chains destined for degradation.

Since ubiquitination is the signal that marks polypeptides including nascent CFTR (Ward *et al.*, 1995) for proteolysis by the proteasome, we determined whether the ansamycin effect might be on ubiquitination. To do this, cells expressing wild-type CFTR were transfected with a plasmid coding for ubiquitin tagged with a hemagglutinin (HA) epitope (Treier *et al.*, 1994). Following immunoprecipitation with an antibody to this epitope, ubiquitinated CFTR could be visualized by immunoblotting (Figure 2). Both geldanamycin-treated and untreated cells revealed ubiquitinated CFTR bands forming a smear reaching to the top of the gel. The ansamysin did not increase the amounts of these. Hence, it would seem that the drug must promote the access of the nascent chains to the proteasome or enhance its action in some other manner rather than increasing ubiquitination.

Ansamycins do not influence the biosynthetic processing of the structurally related P-gp and MRP

Because these drugs had profound effects on the fate of both wild-type and mutant nascent CFTR, we determined whether these were general effects which would be felt by other structurally related membrane glycoproteins. Similar pulse–chase experiments were performed with cells expressing P-glycoprotein or the multidrug resistance-associated protein (MRP) (Figure 3). In contrast to CFTR, these drug transporters mature efficiently both in the absence and presence of geldanamycin treatment. Neither the rates of disappearance of the lower molecular weight precursor bands nor the appearance of the larger

product bands were significantly altered by the drug. Although there is some experimental variability as indicated by the plots in Figure 3, it is clear that the biosynthetic processing of these two membrane glycoproteins is essentially insensitive to geldanamycin. Not only are their wild-type nascent chains not subject to proteasomal degradation, but they are also not made susceptible by ansamycin treatment. On this basis, it would seem that nascent membrane proteins not normally subjected to proteolysis at the ER are not influenced by these compounds.

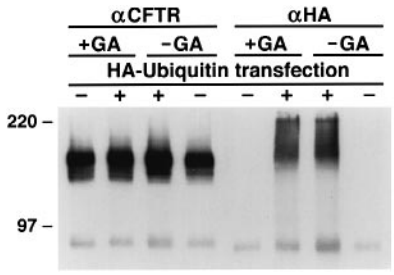

Fig. 2. Geldanamycin does not cause increased ubiquitination of CFTR. BHK cells stably expressing wild-type CFTR were transfected with a plasmid containing the sequence for HA-tagged ubiquitin (Treier *et al.*, 1994). After a 90 min exposure to geldanamycin (+GA), lysates were immunoprecipitated with a polyclonal rabbit antibody to a C-terminal CFTR sequence (Kartner *et al.*, 1991). The immunoprecipitates were electrophoresed and analyzed by Western blotting using monoclonal antibodies to CFTR and the HA epitope as indicated above the lanes. The upper bars above the lanes indicate that the left half of the blot was probed with antibody to CFTR and the right half with antibody to the HA epitope fused to the ubiquitin sequence; the middle set of bars indicate whether cells were treated (+) or not (–) with geldanamycin, and the bars closest to the lanes indicate whether cells were transfected (+) or not (–) with the HA-ubiquitin plasmid. The ubiquitinated CFTR species of increasing size were unaltered by geldanamycin.

Ansamycin-sensitive association of nascent CFTR with Hsp90 but not Grp94

Since cytosolic Hsp90 and its homolog, Grp94, in the ER lumen recently have been shown to be the specific targets of action of the ansamycin benzoquinone drugs (Chavany *et al.*, 1996; Prodromou *et al.*, 1997; Stebbins *et al.*, 1997), it seemed likely that their influence on nascent CFTR might be mediated via one of these chaperones. It was first determined whether either chaperone interacted with nascent CFTR. The results of co-immunoprecipitation experiments such as those illustrated in Figure 4A indicated that Hsp90 does interact while Grp94 apparently does not. Thus, after metabolic labeling with [^{35}S]methionine, a band with the same mobility as nascent CFTR is immunoprecipitated with Hsp90 by antibodies against the chaperone (Figure 4A). On the other hand, no such band was detected on immunoprecipitation of Grp94. As a control, immunoprecipitation was also performed with antibodies to Hsp70 which previously was shown to bind nascent CFTR (Yang *et al.*, 1993). Indeed the same nascent CFTR band was co-immunoprecipitated as with antibodies to Hsp90. Importantly, when cells were treated with geldanamycin, co-immunoprecipitation of the immature CFTR band with Hsp70 was not altered whereas that with Hsp90 was totally ablated. Hence, nascent CFTR associates with Hsp90 in a geldanamycin-sensitive fashion during biosynthesis.

To confirm that the band co-immunoprecipitated with Hsp90 and having the same electrophoretic mobility as immature CFTR was indeed nascent CFTR, both sequential immunoprecipitation and immunoprecipitation followed by immunoblotting experiments were performed. Figure 4B shows that the pulse-labeled immature CFTR band is present in immunoprecipitates obtained with antibody to Hsp90 because when these are dissociated and reprecipitated with M3A7 this band is detected (lane 6); with

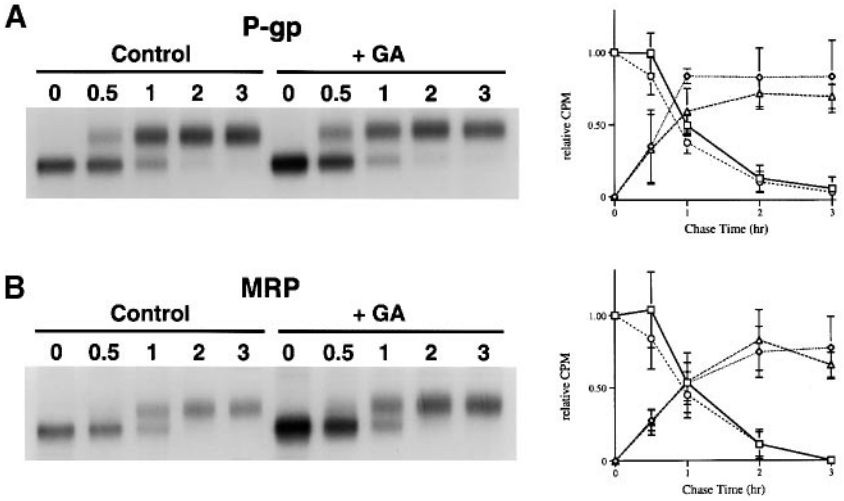

Fig. 3. Effect of geldanamycin on the biosynthetic processing of P-glycoprotein (P-gp) and multidrug resistance protein (MRP). (**A**) Maturation of P-gp in CHO cells in the absence and presence of geldanamycin. The experiments was performed identically to those in Figure 1, with immunoprecipitation using the P-gp-specific monoclonal antibody C-219 (2 μg; Kartner *et al.*, 1985). The graph to the right of the fluorogram illustrates quantitatively the time course of disappearance of the smaller immature band and appearance of the larger mature band. The relative amounts of ^{35}S radioactivity in each band were determined by electronic autoradiography using a Packard Instant Imager. The mean values from three independent experiments are plotted, with the ranges indicated. (**B**) Maturation of MRP in BHK cells with and without geldanamycin treatment. Immunoprecipitation was with the MRP-specific monoclonal antibody, MRPr1 (1 μg; Flens *et al.*, 1994). The graph represents the results of three such experiments with MRP exactly as in (A) using P-gp.

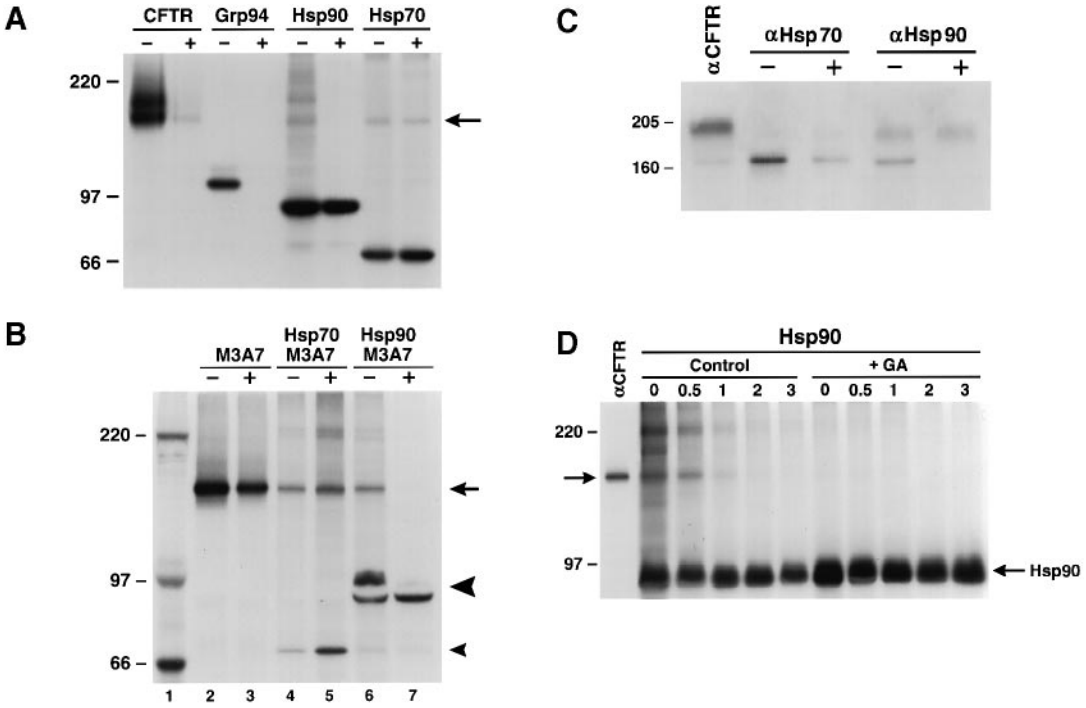

Fig. 4. Association of immature, core-glycosylated CFTR with molecular chaperones. (**A**) Co-immunoprecipitation of nascent CFTR with molecular chaperones. BHK cells, in 60 mm dishes, stably expressing wild-type CFTR were labeled continuously with [^{35}S]methionine for 90 min and then solubilized in 1 ml of NP-40 lysis buffer. For immunoprecipitation, antibodies to CFTR (2 μl M3A7 ascites fluid), Grp94 (Stressgen #850), Hsp90 (10 μl Hsp90–10) and Hsp70 (8 μl Stressgen #815) were added to 1 ml of lysate and incubated overnight at 4°C with gentle rocking. Then 25 μl of protein G–agarose beads were added and incubation continued for an additional 3 h. After four washes with lysis buffer, beads were eluted with gel loading buffer for electrophoresis. The fluorogram shown was obtained by exposure of the dried gel to X-ray film. The (+) and (–) signs indicate that the cells were or were not treated with geldanamycin as described in Materials and methods. The arrow indicates the position of the nascent core-glycosylated CFTR band. It is evident that the immature CFTR band is co-immunoprecipitated with both Hsp90 and Hsp70 but not with Grp94. (**B**) Sequential immunoprecipitation of chaperones Hsp90 and Hsp70 and nascent CFTR. BHK cells stably expressing CFTR in 60 mm culture dishes were pulse-labeled with [^{35}S]methionine (1 μCi/ml) for 30 min and solubilized in 1 ml of NP-40 lysis buffer. For immunoprecipitation, antibodies to CFTR (2 μl M3A7 ascites fluid), Hsp70 (8 μl Stressgen #815) or Hsp90 (10 μl Hsp90–10) were added to 1 ml of lysate and incubated overnight at 4°C with gentle rocking. Protein G–agarose beads (25 μl packed volume) were added and incubation continued for a further 3 h. Following four washes with lysis buffer, the M3A7-bound beads were eluted with gel loading buffer and electrophoresed (lanes labeled M3A7). The beads with antibodies to Hsp70 or Hsp90 were eluted in 1% SDS for 1 h at room temperature. These eluates were then adjusted to the composition of RIPA lysis buffer for a second immunoprecipitation with M3A7. Following RIPA washing, the final protein G–agarose beads were eluted with gel loading buffer and electrophoresed (lanes labeled Hsp70/M3A7 and Hsp90/M3A7). The dried gel was exposed to X-ray film to yield the fluorogram shown, The (+) and (–) signs indicate that the cells were or were not treated with geldanamycin as described in Materials and methods. The arrow indicates the position of the nascent CFTR band; the large arrowhead, Hsp90; and the small arrowhead, Hsp70. (**C**) CFTR detection by immunoblotting of chaperone immunoprecipitates. NP-40 lysates of CFTR-expressing BHK cells were electrophoresed directly (αCFTR lane) together with immunoprecipitates obtained using antibodies to Hsp70 or Hsp90. Following transfer to nitrocellulose, the blot was probed with the M3A7 antibody to CFTR. (+) and (–) signs indicate that cells were or were not treated for 90 min with geldanamycin. (**D**) Association of immature CFTR with Hsp90 during a pulse–chase experiment is abrogated by geldanamycin. A pulse–chase experiment with CFTR-expressing BHK cells was performed as in Figure 1 except that immunoprecipitations were with antibodies to Hsp90 (10 μl Hsp90–10; Smith *et al.*, 1993). The arrow indicates the Hsp90 band.

geldanamycin-treated cells, however, it is not (lane 7). The association of nascent CFTR with Hsp70 has been documented previously (Yang *et al.*, 1993) and is reflected similarly in this sequential immunoprecipitation protocol (Figure 4B, lanes 4 and 5). Consistent with the observations in Figure 4A, geldanamycin was without effect on this association. Figure 4C demonstrates that Western blotting with antibody to CFTR also detects the presence of CFTR bands in both Hsp70 and Hsp90 immunoprecipitates.

In a further attempt to determine if the more rapidly degraded population of nascent CFTR molecules in cells treated with geldanamycin could be detected in association with Hsp90, a pulse–chase experiment was carried out with immunoprecipitation by antibodies to Hsp90 (Figure 4D). No nascent CFTR band was detected in these precipitates even during the pulse labeling (0 chase time), indicating that degradation occurred very quickly when Hsp90 association was perturbed by the drug.

Effects of geldanamycin on rates of synthesis and steady-state amounts of CFTR and chaperones

The data in Figure 1 had shown that the major effect of the ansamycins was to increase turnover of immature CFTR. However, the amount pulse-labeled in the presence of the drug appeared to be slightly reduced as well. Therefore, it was necessary to compare the rates of synthesis in the presence and absence of drug. Furthermore, although it did not appear from Figure 4 that geldanamycin had much influence on the amounts of radiolabeled Hsp90 or Hsp70, it was important to confirm that the apparent decrease in Hsp90-associated CFTR did not reflect an effect of the drug on either the synthesis or turnover of the chaperone. In addition, although we had not found evidence of association of nascent CFTR with Grp94, it was necessary to explain the apparent absence of immunoprecipitable Grp94 in geldanamycin-treated cells (Figure 4A). The synthesis of CFTR and the two

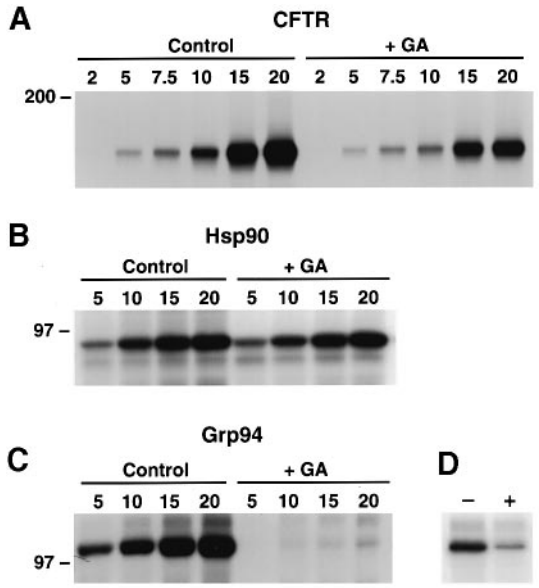

Fig. 5. Rates of synthesis of CFTR, Hsp90 and Grp94 in the absence (control) and presence of geldanamycin (GA). CFTR-expressing BHK cells on 6 cm plates were starved of methionine as in the pulse–chase experiments and then incubated in the presence of [^{35}S]methionine (100 μCi/ml) for the times indicated in minutes prior to immunoprecipitation with M3A7 as in the other experiments. (**A**) Geldanamycin causes a slight reduction in CFTR synthesis. (**B**) Geldanamycin is without influence on the rate of Hsp90 synthesis. (**C**) The Grp94 band in nearly undetectable in immunoprecipitates from geldanamycin-treated cells. (**D**) Geldanamycin greatly reduces the ability of the antibody to Grp94 to immunoprecipitate the protein. Following [^{35}S]methionine labeling for 5 min in the absence of drug, immunoprecipitation was performed in the absence (–) or presence (+) of the drug.

chaperones was followed over 20 min (Figure 5). Geldanamycin did cause a minor decrease in the rate of appearance of labeled nascent CFTR (Figure 5A). However, this effect was small in comparison with the gross increase in its rate of turnover and complete inhibition of maturation. The synthesis of Hsp90 was unaffected by geldanamycin (Figure 5B). Strikingly, as had been seen after longer term [^{35}S]methionine labeling (Figure 4A), the amount of labeled Grp94 formed during synthesis was barely detectable (Figure 5C). However, we considered it unlikely that the synthesis of this chaperone was completely inhibited by the ansamycin and assessed the alternative possibility that the drug interfered with its immunoprecipitation. Figure 5D showed that this latter possibility was indeed the case. A lysate of cells pulse-labeled for 5 min in the absence of drug was then immunoprecipitated in the absence (–) or presence (+) of the drug. Clearly the ability of the antibody to Grp94 to immunoprecipitate this chaperone is greatly reduced by the drug. Of course this means that the rate of synthesis in the presence of geldanamycin cannot be determined from this experiment. Fortunately, however, since Grp94 was found to not bind nascent CFTR in our experiments, it is unlikely to be involved in its processing. Furthermore, steady-state levels of the chaperone are not reduced by geldanamycin (Figure 7D).

To see if an influence of geldanamycin on ER-localized nascent CFTR but not on the mature form at the cell surface could be visualized directly, the localization of CFTR tagged with green fluorescent protein (GFP) was observed. Figure 6 shows that the drug caused the complete disappearance of intracellular, primarily perinuclear, fluorescence in both wild-type and ΔF508 CFTR-expressing cells. The surface fluorescence of the cells expressing the wild-type protein, detectable as increased intensity at the perimeters and a lighter tent-like veil over the entire surface, is retained after drug treatment.

To determine the impact of the large increase in nascent CFTR degradation, caused by geldanamycin, on the amount of mature CFTR in cells, Western blots were performed (Figure 7A). Up until 8 h after drug treatment, little decrease in amount was detected; by 24 h, there was a substantial decrease. These are the results which would be expected if only the turnover of the immature translation product were influenced. The resulting reduction in formation of its mature product would only become apparent as a diminished steady-state amount after times comparable with the $t_{1/2}$ of its turnover which is estimated at 10–20 h (Lukacs *et al.*, 1994; Ward and Kopito, 1994). The ansamycins are known to have secondary effects beyond their disruption of Hsp90/Grp94–substrate interactions, including the induction of stress proteins (Lawson *et al.*, 1998). Therefore, we assessed by Western blotting changes in the steady-state levels of Hsp90 and Grp94 as well as the other two molecular chaperones, known to bind nascent CFTR, Hsp70 and calnexin. Substantial increases in the quantity of Grp94 occurred progressively over several hours (Figure 7D). Hsp70 showed a similar but less pronounced elevation (Figure 7C). There may have been a slight increase in Hsp90 amount, but this is barely detectable (Figure 7B). The calnexin signals appeared essentially unchanged (Figure 7E). None of the changes which did occur were early enough to account for the very rapid increase in nascent CFTR turnover (Figures 1 and 4).

Discussion

Two molecular chaperones had already been shown to interact with nascent CFTR (Yang *et al.*, 1993; Pind *et al.*, 1994), one on each side of the ER membrane. In this study, we have demonstrated that the immature CFTR protein also complexes with the most abundant cytosolic chaperone, Hsp90. Furthermore, this interaction was found to have a major impact on the fate of the nascent CFTR polypeptide as it is being processed at the ER membrane. Disruption of its normal interaction with Hsp90 by the ansamycin drugs greatly accelerates its rate of degradation and nearly completely abrogates its maturation. The promotion of proteasomal degradation of an incomplete or imperfect cytoplasmic protein seems to be a common consequence of interfering with the protein's association with Hsp90 (Tsubuki *et al.*, 1994; Wagner and Margolis, 1995).

Multiple steroid hormone receptors (Pratt and Toft, 1997) and several other transcription factors (Wilhelmsson *et al.*, 1990; Holley and Yamamoto, 1995; Nair *et al.*, 1996), as well as a large number of tyrosine kinases (Oppermann *et al.*, 1981; Hartson and Matts, 1994) and some other proteins having a major impact on cell proliferation (Sepehrnia *et al.*, 1996; Dasgupta and Momand, 1997), are all subjected to proteolysis by the proteasome when Hsp90 function is perturbed. Interactions

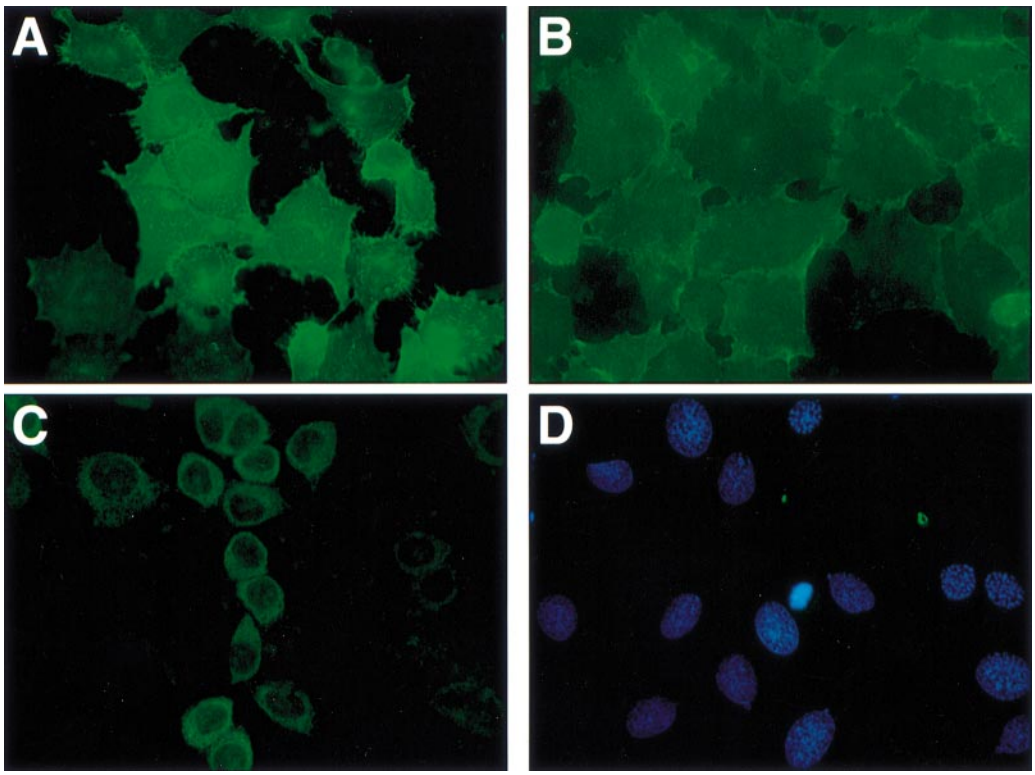

Fig. 6. Localization at the surface and intracellularly in BHK cells expressing wild-type and ΔF508 CFTR fused to GFP at their C-termini. An FITC filter was employed for the fluorescence microscopy. (**A**) The GFP fusion with wild-type CFTR is observed both over the entire cell surface and also in a perinuclear distribution which can be seen as intensifications below the uniform surface veil. (**B**) After treatment with geldanamycin, the surface localization of the wild-type is unchanged but the perinuclear image is no longer seen. (**C**) The ΔF508 CFTR fusion with GFP forms only the perinuclear images. (**D**) After gendanamycin treatment, the ΔF508 CFTR fusion with GFP is undetectable. Nuclei were stained with DAPI.

of Hsp90 and components of the ubiquitin–proteasome pathway with all these substrates probably occur in the cytoplasm. At least in the case of the ubiquitination and proteolysis by the proteasome, however, a great deal of recent attention has focused on its crucial participation in the overall ER quality control system which scrutinizes both membrane and secretory proteins during their biogenesis at the ER (Sommer and Wolf, 1997). Thus all or portions of such molecules detected as misfolded in the ER lumen may be 'retrotranslocated' back through the translocon to the cytoplasmic surface of the ER where they are ubiquitinated and degraded by the proteasome (Werner *et al.*, 1996). Although participation of several luminal and ER membrane components of this recognition and reverse translocation system have been demonstrated (Wiertz *et al.*, 1996), cytosolic participants other than those involved in ubiquitination and proteolysis have not been identified.

Our observations that an interaction of Hsp90 with cytoplasmic aspects of nascent CFTR counteracts its susceptibility to proteasomal degradation may suggest that this chaperone could participate at a late stage in the overall ER quality control mechanism. It will be of interest to test whether the aberrant secretory pathway products found to be retrotranslocated (Qu *et al.*, 1996) are also influenced by Hsp 90 prior to or during proteolysis. There is as yet no direct evidence as to whether or not CFTR undergoes any retrotranslocation prior to the proteasomal digestion of its large cytoplasmic domains. According to current topological models (Chang *et al.*, 1994), very little of the polypeptide is exposed at the luminal surface. However, the nascent molecules which eventually do succumb to the proteasome have already been core glycosylated on the second of only two substantial extracytoplasmic loops (more than ~10 residues in length) in the protein. Calnexin has interacted with these oligosaccharides prior to proteolysis (Ward and Kopito, 1994).

If the large cytoplasmic domains were cleaved processively, essentially to completion, by the proteasome, there is currently no insight into what the fate of the six residual 'hairpin' structures, each consisting of two transmembrane segments and a short intervening hydrophilic 'loop', might be. On the other hand, a great deal of conjecture is possible regarding events at the cytoplasmic surface. One recent report (Biederer *et al.*, 1997) has claimed that the ER surface may serve as a 'platform' to which even some defective soluble cytoplasmic proteins may be recruited to be ubiquitinated and degraded by the proteasome. Again in view of our current observations, it will be of interest to determine whether Hsp90 plays any role in the handling of these proteins. If it does, it might be considered a point of confluence of pathways of quality control of both cytoplasmic and secretory proteins. Alternatively, the cytoplasmic domains of CFTR may simply be dealt with as are the bulk of soluble cytoplasmic proteins which are not tethered to the ER surface. In this scenario, such cytoplasmic quality control might or might not be coordinated with ER quality control.

In fact there is no direct evidence that major players in

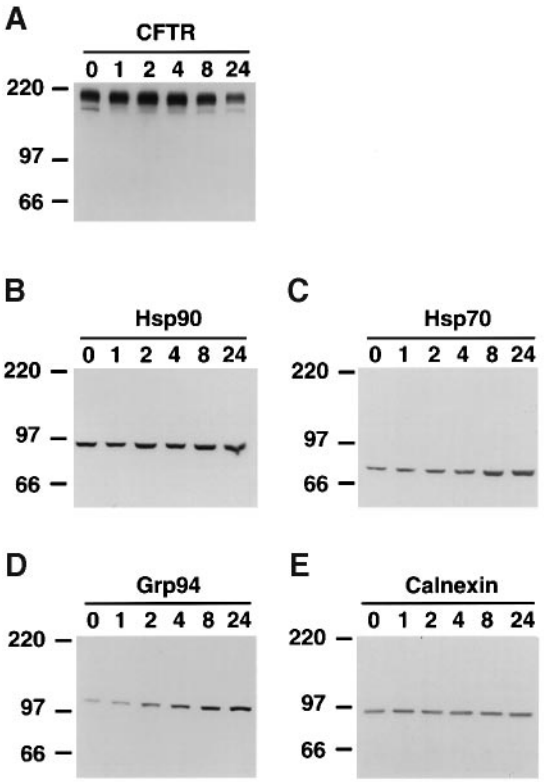

Fig. 7. Influence of geldanamycin on the steady-state levels of CFTR and molecular chaperones in CFTR-expressing BHK cells. Cells were exposed to geldanamycin (0.1 μg/ml) for the times indicated in hours. Whole cell lysates were electrophoresed and analyzed by Western blots probed with antibodies to each of the proteins. The secondary antibody was anti-mouse Ig conjugated with horseradish peroxidase. Chemiluminescent film detection utilized the ECL kit (Amersham). (**A**) The amounts of mature CFTR (upper band) and the small amount of an alternatively initiated form (lower band) are reduced slightly by 8 h and substantially by 24 h. (**B**) The amount of Hsp90 increases slightly over this time course. (**C**) There is a considerable increment in the amount of Hsp70, most evident at 8 and 24 h. (**D**) The ER chaperone, Grp94, increases most due to geldanamycin treatment. (**E**) The amount of calnexin does not change during this period.

ER quality control such as calnexin (Hebert *et al.*, 1995) and Bip (Morris *et al.*, 1997) influence CFTR processing. Although calnexin binding occurs (Pind *et al.*, 1994), its blockage does not either prevent the wild-type molecule from being transported to the surface or allow the ΔF508 molecule to do so. We have been unable to detect association of wild-type nascent CFTR with Bip. Of particular relevance to the present study, we were also unable to detect any interaction with Grp94, the homolog of Hsp90 present in the ER lumen. This is significant because the only other membrane protein whose turnover has been shown to be sensitive to the ansamycin drugs is p185erb-B2, which does bind Grp94 (Chavany *et al.*, 1996). Proteasomal degradation of both its nascent and mature forms is enhanced by ansamycin disruption of its interaction with Grp94 (Mimnaugh *et al.*, 1996). Topologically, the involvement of Grp94 rather than Hsp90 corresponds well with the fact that a large portion of this single-pass membrane protein is located extracytoplasmically, and the proteasomal digestion of at least the nascent chain would seem to require retrotranslocation.

The turnover of the more closely related polytopic membrane proteins, P-gp and MRP, is entirely insensitive to ansamycins. In contrast to CFTR, the wild-type versions of these molecules mature efficiently and their nascent chains are not proteolysed (Figure 3; Loo and Clarke, 1997). While we have not yet tested directly their association with Hsp90, for the moment, we interpret their insensitivity to ansamycins as indicating that an association does not occur. This is consistent with the notion that Hsp90 and probably its partners (Jakob and Buchner, 1994; Schneider *et al.*, 1996) are involved in the processing of incompletely folded or assembled cytoplasmic proteins or domains but not of those which have achieved a complete native state. It remains to be determined if this is generally true for incompletely assembled cytoplasmic domains of large integral membrane proteins as well as soluble cytosolic proteins and possibly even aberrant secretory products retrotranslocated to the cytoplasmic surface of the ER.

It will also be important to ascertain which other cytoplasmic chaperones and cofactors contribute to CFTR processing. Among the obvious candidates to be tested are those which participate in a large dynamic Hsp90-centered complex required for steroid hormone receptor maturation (Nair *et al.*, 1996; Dittmar and Pratt, 1997; Pratt and Toft, 1997). Preliminary observations indicate that the p23 partner protein of Hsp 90 is also present in a complex of the chaperone with nascent CFTR. Once other members are identified, extensive kinetic analyses will be required to illuminate the sequence of events in the chaperoning and avoidance of proteolysis of nascent CFTR.

Materials and methods

Cells

The CHO and BHK cell lines expressing wild-type and ΔF508 CFTR have been described in our earlier publications (Chang *et al.*, 1993; Seibert *et al.*, 1995). BHK cells stably expressing a CFTR–GFP fusion were established and characterized as described elsewhere (T.J.Jensen, L.Cui, Y.-X.Hou, X.-B.Chang and J.R.Riordan, in preparation). The CHO cell line expressing P-glycoprotein originated from the work of Kartner *et al.* (1985). MRP-expressing BHK cells are those recently described by Chang *et al.* (1997). Conditions for the culture of these cells were also as described in these references.

Antibodies

The monoclonal antibody which recognizes an epitope between residues 1365 and 1394 of CFTR was prepared by Kartner *et al.* (1992). Monoclonal antibodies to P-glycoprotein and MRP were C-219 (Kartner *et al.*, 1985) and MRPr1 (Flens *et al.*, 1994), respectively. Antibodies to the chaperones studied were obtained as follows: Hsp90 (H90-10, David Toft, Mayo Clinic); Hsp70 (Stressgen); Grp94 (Stressgen).

Metabolic labeling with [^{35}S]methionine

Cells were starved of methionine for 30 min by incubation in methionine-free medium and then labeled for the periods indicated in the figure legends with 100 μCi/ml of [^{35}S]methionine (>800 Ci/mmol; Amersham). In experiments where a chase followed, the isotope-containing medium was replaced by complete medium containing 7% fetal bovine serum and 1 mM methionine. To evaluate the influence of the ansamycins, geldanamycin (Drug Synthesis and Chemistry Branch, NCI) or herbimycin A (LC Labs) and the proteasome inhibitor, lactacystin (E.J.Corey, Department of Chemistry, Harvard University), cells were generally pre-incubated with the concentration of these compounds indicated in the figure legends for 90 min prior to methionine starvation.

Cell lysis and immunoprecipitation

Cells were lysed using a solution of 50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 20 mM $NaMoO_4$ and 0.09% N-P40 containing the following protease inhibitors: E64 (3.5 μg/ml), benzamidine (100 μg/ml), aprotinin

(5 μg/ml), leupeptin (10 μg/ml) and Pefabloc (50 μg/ml). Following centrifugation at 4°C at 15 000 *g* for 15 min, the supernatant was incubated overnight with the appropriate primary antibody. Protein G–agarose (Gibco-BRL) was then used to remove the complexes formed. After four washings of the beads with lysis buffer, the attached complexes were dissolved in electrophoresis sample buffer.

SDS–PAGE and immunoblotting

Either these immunoprecipitates or total cell lysates were electrophoresed on 7% acrylamide gels which were run and prepared for fluorography as described previously (Kartner *et al.*, 1991). For Western blotting, transfer to nitrocellulose membranes (Bio-Rad), probing with the antibodies specified in the figure legends and detection by enhanced chemiluminescence (Amersham) were also according to protocols we have detailed before (Seibert *et al.*, 1996). After electrophoresis of immunoprecipitates, gels were dried and analyzed by fluorography and electronic autoradiography using a Packard Instant Imager.

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References

Biederer,T., Volkwein,C. and Sommer,T. (1997) Role of Cue1p in ubiquitination and degradation at the ER surface. *Science*, **278**, 1806–1809.

Buchner,J. (1996) Supervising the fold: functional principles of molecular chaperones. *FASEB J.*, **10**, 10–19.

Chang,X.-B., Tabcharani,J.A., Hou,Y.-X., Jensen,T.J., Kartner,N., Alon,N., Hanrahan,J.W. and Riordan,J.R. (1993) Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all ten PKA consensus phosphorylation sites. *J. Biol. Chem.*, **268**, 11304–11311.

Chang,X.-B., Hou,Y.-X., Jensen,T. and Riordan,J.R. (1994) Mapping of the cystic fibrosis transmembrane conductance regulator membrane topology by glycosylation site insertion. *J. Biol. Chem.*, **269**, 18572–18575.

Chang,X.-B., Hou,Y.X. and Riordan,J.R. (1997) ATPase activity of purified multidrug resistance-associated protein. *J. Biol. Chem.*, **272**, 30962–30968.

Chavany,C. *et al*. (1996) p185erbB2 binds to GRP94 *in vivo*. Dissociation of the p185erbB2/GRP94 heterocomplex by benzoquinone ansamycins precedes depletion of p185erbB2. *J. Biol. Chem.*, **271**, 4974–4977.

Chen,W., Helenius,J., Braakman,I. and Helenius,A. (1995) Cotranslational folding and calnexin binding during glycoprotein synthesis. *Proc. Natl Acad. Sci. USA*, **92**, 6229–6233.

Cheng,S.H., Rich,D.P., Marshall,J., Gregory,R.J., Welsh,M.J. and Smith,A.E. (1991) Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell*, **66**, 1027–1036.

Collins,F.S. (1992) Cystic fibrosis: molecular biology and therapeutic implications. *Science*, **256**, 774–779.

Dasgupta,G. and Momand,J. (1997) Geldanamycin prevents nuclear translocation of mutant p53. *Exp. Cell Res.*, **237**, 29–37.

Dittmar,K.D. and Pratt,W.B. (1997) Folding of the glucocorticoid receptor by the reconstituted Hsp90-based chaperone machinery. The initial hsp90·p60·hsp70-dependent step is sufficient for creating the steroid binding conformation. *J. Biol. Chem.*, **272**, 13047–13054.

Fenteany,G., Standaert,R.F., Lane,W.S., Choi,S., Corey,E.J. and Schreiber,S.L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science*, **268**, 726–731.

Flens,M.J., Izquierdo,M.A., Scheffer,G.L., Fritz,J.M., Meijer,C.J., Scheper,R.J. and Zaman,G.J. (1994) Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res.*, **54**, 4557–4563.

Frydman,J. and Hohfeld,J. (1997) Chaperones get in touch: the Hip–Hop connection. *Trends Biochem. Sci.*, **22**, 87–92.

Grenert,J.P. *et al*. (1997) The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J. Biol. Chem.*, **272**, 23843–23850.

Hartson,S.D. and Matts,R.L. (1994) Association of Hsp90 with cellular Src-family kinases in a cell-free system correlates with altered kinase structure and function. *Biochemistry*, **33**, 8912–8920.

Hebert,D.N., Foellmer,B. and Helenius,A. (1995) Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell*, **81**, 425–433.

Holley,S.J. and Yamamoto,K.R. (1995) A role for Hsp90 in retinoid receptor signal transduction. *Mol. Biol. Cell*, **6**, 1833–1842.

Jakob,U. and Buchner,J. (1994) Assisting spontaneity: the role of Hsp90 and small Hsps as molecular chaperones. *Trends Biochem. Sci.*, **19**, 25.

Jensen,T.J., Loo,M.A., Pind,S., Williams,D.B., Goldberg,A.L. and Riordan,J.R. (1995) Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell*, **83**, 129–135.

Johnson,J.L. and Craig,E.A. (1997) Protein folding *in vivo*: unraveling complex pathways. *Cell*, **90**, 201–204.

Kartner,N., Evernden-Porelle,D., Bradley,G. and Ling,V. (1985) Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature*, **316**, 820–823.

Kartner,N. *et al*. (1991) Expression of the cystic fibrosis gene in non-epithelial invertebrate cells produces a regulated anion conductance. *Cell*, **64**, 681–691.

Kartner,N., Augustinas,O., Jensen,T.J., Naismith,A.L. and Riordan,J.R. (1992) Mislocalization of delta F508 CFTR in cystic fibrosis sweat gland. *Nature Genet.*, **1**, 321–327.

Lawson,B., Brewer,J.W. and Hendershot,L.M. (1998) Geldanamycin, an hsp90/GRP94-binding drug, induces increased transcription of endoplasmic reticulum (ER) chaperones via the ER stress pathway. *J. Cell Physiol.*, **174**, 170–178.

Loo,T.W. and Clarke,D.M. (1997) Correction of defective protein kinesis of human P-glycoprotein mutants by substrates and modulators. *J. Biol. Chem.*, **272**, 709–712.

Lukacs,G.L., Mohamed,A., Kartner,N., Chang,X.-B., Riordan,J.R. and Grinstein,S. (1994) Conformational maturation of CFTR but not its mutant counterpart (ΔF508) occurs in the endoplasmic reticulum and requires ATP. *EMBO J.*, **13**, 6076–6086.

Mimnaugh,E.G., Chavany,C. and Neckers,L. (1996) Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. *J. Biol. Chem.*, **271**, 22796–22801.

Morris,J.A., Dorner,A.J., Edwards,C.A., Hendershot,L.M. and Kaufman, R.J. (1997) Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. *J. Biol. Chem.*, **272**, 4327–4334.

Nair,S.C., Toran,E.J., Rimerman,R.A., Hjermstad,S., Smithgall,T.E. and Smith,D.F. (1996) A pathway of multi-chaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor Hsf1 and the aryl hydrocarbon receptor. *Cell Stress Chaperones*, **1**, 237–250.

Oppermann,H., Levinson,W. and Bishop,J.M. (1981) A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat-shock protein. *Proc. Natl Acad. Sci. USA*, **78**, 1067–1071.

Pind,S., Riordan,J.R. and Williams,D.B. (1994) Participation of the endoplasmic reticulum chaperone calnexin (p88, IP90) in the biogenesis of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.*, **269**, 12784–12788.

Pratt,W.B. and Toft,D.O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.*, **18**, 306–360.

Prodromou,C., Roe,S.M., O'Brien,R., Ladbury,J.E., Piper,P.W. and Pearl,L.H. (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell*, **90**, 65–75.

Qu,B.H. and Thomas,P.J. (1996) Alteration of the cystic fibrosis transmembrane conductance regulator folding pathway. *J. Biol. Chem.*, **271**, 7261–7264.

Qu,D., Teckman,J.H., Omura,S. and Perlmutter,D.H. (1996) Degradation of a mutant secretory protein, α1-antitrypsin Z, in the endoplasmic reticulum requires proteasome activity. *J. Biol. Chem.*, **271**, 22791–22795.

Qu,B.H., Strickland,E.H. and Thomas,P.J. (1997) Localization and suppression of a kinetic defect in cystic fibrosis transmembrane conductance regulator folding. *J. Biol. Chem.*, **272**, 15739–15744.

Scheibel,T., Weikl,T. and Buchner,J. (1998) Two chaperone sites in Hsp90 differing in substrate specificity and ATP dependence. *Proc. Natl Acad. Sci. USA*, **95**, 1495–1499.

Schneider,C., Sepp-Lorenzino,L., Nimmesgern,E., Ouerfelli,O., Danishefsky,S., Rosen,N. and Hartl,F.U. (1996) Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proc. Natl Acad. Sci. USA*, **93**, 14536–14541.

Seibert,F.S., Tabcharani,J.A., Chang,X.-B., Dulhanty,A.M., Mathews,C., Hanrahan,J.W. and Riordan,J.R. (1995) cAMP-dependent protein kinase-mediated phosphorylation of cystic fibrosis transmembrane conductance regulator residue ser-753 and its role in channel activation. *J. Biol. Chem.*, **270**, 2158–2162.

Seibert,F.S., Linsdell,P., Loo,T.W., Hanrahan,J.W., Clarke,D.M. and Riordan,J.R. (1996) Disease-associated mutations in the fourth cytoplasmic loop of CFTR compromise biosynthetic processing and chloride channel activity. *J. Biol. Chem.*, **271**, 15139–15143.

Sepehrnia,B., Paz,I.B., Dasgupta,G. and Momand,J. (1996) Heat shock protein 84 forms a complex with mutant p53 protein predominantly within a cytoplasmic compartment of the cell. *J. Biol. Chem.*, **271**, 15084–15090.

Sherman,M.Y. and Goldberg,A.L. (1996) Involvement of molecular chaperones in intracellular protein breakdown. *Experientia Suppl.*, **77**, 57–78.

Smith,D.F., Sullivan,W.P., Marion,T.N., Zaitsu,K., Madden,B., McCormick,D.J. and Toft,D.O. (1993) Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. *Mol. Cell. Biol.*, **13**, 869–876.

Smith,D.F., Whitesell,L., Nair,S.C., Chen,S., Prapapanich,V. and Rimerman,R.A. (1995) Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. *Mol. Cell. Biol.*, **15**, 6804–6812.

Sommer,T. and Jentsch,S. (1993) A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. *Nature*, **365**, 176–179.

Sommer,T. and Wolf,D.H. (1997) Endoplasmic reticulum degradation: reverse protein flow of no return. *FASEB J.*, **11**, 1227–1233.

Stebbins,C.E., Russo,A.A., Schneider,C., Rosen,N., Hartl,F.U. and Pavletich,N.P. (1997) Crystal structure of an Hsp90–geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell*, **89**, 239–250.

Treier,M., Staszewski,L.M. and Bohmann,D. (1994) Ubiquitin-dependent c-Jun degradation *in vivo* is mediated by the δ domain. *Cell*, 787–798.

Tsubuki,S., Saito,Y. and Kawashima,S. (1994) Purification and characterization of an endogenous inhibitor specific to the Z-Leu-Leu-Leu-MCA degrading activity in proteasome and its identification as heat-shock protein 90. *FEBS Lett*, **344**, 229–233.

Wagner,B.J. and Margolis,J.W. (1995) Age-dependent association of isolated bovine lens multicatalytic proteinase complex (proteasome) with heat-shock protein 90, an endogenous inhibitor. *Arch. Biochem. Biophys.*, **323**, 455–462.

Ward,C.L. and Kopito,R.R. (1994) Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *J. Biol. Chem.*, **269**, 25710–25718.

Ward,C.L., Omura,S. and Kopito,R.R. (1995) Degradation of CFTR by the ubiquitin–proteasome pathway. *Cell*, **83**, 121–127.

Werner,E.D., Brodsky,J.L. and McCracken,A.A. (1996) Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. *Proc. Natl Acad. Sci. USA*, **93**, 13797–13801.

Wiertz,E.J., Tortorella,D., Bogyo,M., Yu,J., Mothes,W., Jones,T.R., Rapoport,T.A. and Ploegh,H.L. (1996) Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature*, **384**, 432–438.

Wilhelmsson,A., Cuthill,S., Denis,M., Wikstrom,A.C., Gustafsson,J.A. and Poellinger,L. (1990) The specific DNA binding activity of the dioxin receptor is modulated by the 90 kd heat shock protein. *EMBO J.*, **9**, 69–76.

Yang,Y., Janich,S., Cohn,J.A. and Wilson,J.M. (1993) The common variant of cystic fibrosis transmembrane conductance regulator is recognized by hsp70 and degraded in a pre-Golgi nonlysosomal compartment. *Proc. Natl Acad. Sci. USA*, **90**, 9480–9484.

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