The human DnaJ homologue (Hdj)-1/heat-shock protein (Hsp) 40 co-chaperone is required for the in vivo stabilization of the cystic fibrosis transmembrane conductance regulator by Hsp70

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INTRODUCTION

The cystic fibrosis (CF) transmembrane conductance regulator (CFTR), the product of the gene that is defective in CF, the most common fatal genetic disease in Caucasians, is a polytopic integral membrane protein that functions as a cAMP-stimulated chloride channel in the apical membrane of epithelial cells [1]. The 1480 amino acid-long polypeptide chain of CFTR includes two transmembrane domains (TM1 and TM2) with six segments each, two nucleotide-binding domains (NBD1 and NBD2) and one cytoplasmic regulatory (R) domain [1]. The most prevalent disease-causing mutation (present on approx. 70% of all CF chromosomes worldwide) is the deletion of a single Phe residue at position 508 (F508del) in NDB1 [1].

CFTR or ABCC7 is a member of the superfamily of ATP-binding cassette (ABC) transporters. In humans, about 50 ABC proteins, grouped into seven different classes, have been identified [2]; see also www.humanabc.org.

As happens with other multi-domain glycoproteins, CFTR is co-translationally inserted into the endoplasmic reticulum (ER) membrane and concomitantly N-linked to glycosyl groups [3]. Generally, addition and trimming of these post-translational modifications, as well as rounds of binding/release with the ER chaperone calnexin, are predicted to facilitate folding through an iterative process until the protein acquires its fully folded native conformation [4]. Only after proper folding, assessed by the ER quality control, can the proteins proceed through the secretory pathway across the Golgi, where they are fully glycosylated [4]. The molecular pathogenesis of several human diseases involves defective intracellular transport of aberrantly folded glycoproteins that are discarded by this conformation-sensitive cellular quality control.

Unlike for instance P-glycoprotein or multidrug resistance-related protein, wild-type (wt) CFTR matures inefficiently, as less than 30% of the protein produced in heterologous expression systems is converted into its fully glycosylated form [5]. F508del causes an even greater CFTR maturation and trafficking defect, as determined by studies on heterologous expression systems, which fail to biochemically observe the respective fully glycosylated form and protein at the cell membrane [6,7]. It is generally believed, although based on indirect evidence, that ER retention of F508del-CFTR is caused by recognition of abnormal folding. Indeed, it was shown that the in vitro folding of a synthetic peptide from the NBD1 of CFTR lacking the F508 residue is kinetically unfavourable relative to folding of the corresponding wt peptide [8]. Moreover, the immature forms of wt-CFTR and

Key words: CFTR, chaperone, endoplasmic reticulum-associated degradation.

Abbreviations used: ABC, ATP-binding cassette; Ab, antibody; BHK, baby hamster kidney; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; CHIP, C-terminus of Hsc70-interacting protein; CHO, Chinese hamster ovary; DSG, deoxyspergualin; ER, endoplasmic reticulum; F508del, deletion of Phe-508; Hdj, human DnaJ homologue; Hsc, heat-shock cognate; Hsp, heat shock; HSF1, heat-shock factor 1; NBD, nucleotide-binding domain; 4-PBA, sodium 4-phenylbutyrate; wt, wild type.

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F508del-CFTR were demonstrated to have different partial proteolytic patterns and hence must correspond to different conformations [9].

In native epithelia, however, F508del-CFTR was described to be apically localized [10] and more recently it was shown that this occurs in a significantly lower proportion of cells than for wt-CFTR [11]. The latter observations are thus consistent with a trafficking defect for F508del-CFTR that is more drastically observed in heterologous expression systems. Also, when correctly localized, F508del-CFTR seems to have at least partial Cl− channel activity [12,13], also confirmed by detection of residual function in native tissues of human origin [14] or from the F508del mouse model [15]. Understanding and monitoring the quality control mechanisms responsible for ER retention of F508del-CFTR may thus be useful to achieve its apical localization, with potential benefit for the management of CF as well as for a number of human diseases involving defective trafficking of misfolded proteins.

The most obvious agents implicated in this cellular quality control are molecular chaperones, due to their capacity to recognize abnormally folded proteins unspecifically, through the exposure of otherwise hidden hydrophobic domains. They are generally known as ‘molecular folders’ but have also been implicated in targeting for degradation, a process tightly linked to the cellular quality control (for recent review see [16]). Both wt- and F508del-CFTR were shown to undergo degradation through the ubiquitin–proteasome pathway [17,18]. On the other hand, the ER-associated degradation of CFTR, seems to be linked to its retrograde translocation, and recently, SSA1, the yeast heat-shock protein (Hsp) 70 orthologue, was shown to facilitate CFTR folding and maturation, namely heat-shock protein 70 (Hsc) 70 [19].

Additionally to the above-mentioned in vitro experiments, several in vivo studies have suggested that molecular chaperones facilitate CFTR folding and maturation, namely heat-shock cognate (Hsc) 70/Hsp70 [20], human DnaJ homologue (Hdj)-2 [21] and Hsp90 [22] in the cytoplasm, and calnexin [23]. The latter ER membrane chaperone was described to transiently bind to the ER (immature) form of CFTR, and appearance of the fully glycosylated mature form was shown to be concomitant with its dissociation. Similarly, complexes of F508del-CFTR with Hsp70/Hsc70 were demonstrated to have different partial processing efficiency of wt- or F508del-CFTR, as an example of a multi-domain co-translationally inserted membrane protein.

Here we tested whether overexpressing Hsp70 alone or with its co-chaperone Hdj-1/Hsp40 causes an effect on the turnover and processing efficiency of wt- or F508del-CFTR, as an example of a multi-domain co-translationally inserted membrane protein.

**EXPERIMENTAL**

**Cells, culture conditions and transfections**

Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cell lines expressing wt- or F508del-CFTR were cultivated as described [30,31].

Hsp70 cDNA was removed from plasmid pH2.3 [32] with BamHI and EcoRI and inserted into pcDNA3 (Invitrogen) after BamHI/EcoRI digestion. Hdj-1/Hsp40 cDNA was removed from pGEX4T-Hdj-1 with BamHI and NotI and inserted into pcDNA3 after BamHI/NotI digestion.

Human Hsp70 cDNA (5 µg) cloned into the pcDNA3 vector was used in transient transfections with Lipofectin® (Life Technologies) or GenePorter® (Gene Therapy Systems). The same amount of non-recombinant pcDNA3 was used to mock-transfect cells as a negative control. In each experiment, cells were co-transfected with 0.5 µg of the pEGFP-C2 plasmid (Clontech) as a control for transfection efficiency. The percentage of cells expressing enhanced green fluorescent protein-C2 was determined at 24 h or 48 h post-transfection by observation under fluorescence inversion microscope and determined to be 35%, on average.

For Hsp70 stable transfections, 2 µg of the above-described pcDNA3-Hsp70 construct was used to transfect BHK cells already stably expressing wt- or F508del-CFTR under methotrexate selection. Following transfection (48 h), cells were trypsinized and selective medium containing 750 µg/ml G418 was added. After 10 days, clones were selected and screened for Hsp70 expression by Western blot as described below. Stable mock transfecants were produced by the same procedure, but using the non-recombinant pcDNA3 vector.

For Hsp70 and Hdj-1/Hsp40 co-expression, 3 µg of each pcDNA3 construct was used in transient transfections with Lipofectin®.

For experiments with 4-PBA, we used a 5 mM treatment with 4-PBA for 48 h to analyse CFTR turnover and processing. Pulse-chase and immunoprecipitation were performed as described below. 4-PBA concentration and incubation time were chosen as those corresponding to the highest decrease in Hsc70 cellular levels by Western blot (results not shown).

For experiments with DSG, we used a 72 h treatment with 50 µg/ml DSG to analyse CFTR turnover and processing. Culture medium was replaced every day to account for DSG degradation. Pulse-chase and immunoprecipitation were performed as described below. DSG concentration and incubation time were those described by other authors [29].

**Antibodies (Abs)**

The following Abs were used: anti-CFTR mouse monoclonal M5A7 Ab, generated against CFTR amino acids 1197–1480 (Chemicon catalogue no. MAB3480); anti-CFTR rabbit polyclonal 169 Ab [33], generated against CFTR amino acids 724–746; rabbit polyclonal anti-CFTR Ab, generated against a glutathione S-transferase fusion protein containing CFTR residues 1–79; polyclonal anti-Hsp70 Ab (StressGen catalogue no. SPA812); monoclonal anti-Hsc70 Ab (StressGen catalogue no. SPA815); polyclonal anti-Hdj1/Hsp40 Ab; polyclonal anti-(heat-shock factor 1) (HSF1) Ab (StressGen catalogue no.
Detection of Hsp70, Hsc70 and Hdj-1/Hsp40 by Western blot

Post-transfection (24 h), about $2 \times 10^6$ cells were lysed with sample buffer [1.5% (w/v) SDS, 5% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue, 0.5 mM dithiothreitol and 31.25 mM Tris, pH 6.8]. Total protein was quantified by a modified micro Lowry method and aliquots of 30 μg of total protein were loaded to 7% (w/v) polyacrylamide mini-gels for electrophoretic separation and transferred onto to nitrocellulose filters. The filters were probed with either anti-Hsp70 or anti-Hdj-1/Hsp40 Abs (see above). As a loading control, blots were re-probed with the anti-α-actin Ab (see above). Blots were developed using the ECL™ detection system (Amersham Bioscience).

Pulse–chase and CFTR immunoprecipitation

Twenty-four hours after transient transfections or 24 h after splitting (for stable lines), cells expressing wt- or F508del-CFTR were starved for 30 min in methionine-free x-modified Eagle’s medium or minimal essential medium and then pulsed for 30 min in the same medium supplemented with 100 μCi/ml [35S]methionine. After chasing for 0, 0.5, 1, 1.5, 2 and 3 h in x-modified Eagle’s medium with 8% (v/v) fetal bovine serum and 1 mM non-radioactive methionine, cells were lysed in 1 ml of RIPA buffer [1% (w/v) deoxycholic acid, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl]. The immunoprecipitation was carried out as described previously [17], using one of the anti-CFTR Abs (see above) in independent experiments and Protein G–agarose or Protein A–Sepharose beads. Immunoprecipitated proteins were eluted from the beads with sample buffer for 1 h at room temperature and then electrophoretically separated on 7% (w/v) polyacrylamide gels. Gels were pre-fixed in methanol/acetic acid (30:10, v/v), washed in water and, for fluorography, soaked in 1 M sodium salicylate for 60 min. After drying at 80 °C for 2 h, gels were exposed to X-ray films ( Biomax; Kodak).

Densitometry

Fluorograms of gels were digitized on a Sharp JX-330 scanner. Integrated peak areas were determined using ImageMaster® software (Amersham Bioscience).

Immunocytochemistry

To determine whether Hsp70 stable transfection induces a continuous stress response, we determined by immunocytochemistry the intracellular localization of HSF1, which migrates to the nucleus under stress conditions [34]. Cells ($2 \times 10^4$) were grown on chamber slides (Nalge Nunc) and were rinsed twice with cold PBS and fixed in methanol for 10 min at $-20^\circ$C, followed by two washes with PBS and incubation in 0.2% (v/v) Triton X-100 for 10 min for cell permeabilization. Unless otherwise stated, all incubations were at room temperature. Non-specific staining was prevented by blocking with 1% (w/v) BSA for 20 min and incubation with anti-HSF1 Ab (diluted 1:100) was for 1 h at room temperature. After three 10 min washes with PBS, cells were incubated with the respective secondary Ab for 1 h, and washed as above. Slides were mounted with Vectashield (Vector Laboratories) containing DAPI (4,6-diamino-2-phenylindole; Sigma Chemical Co.) for nuclei staining and covered with a glass coverslip. Immunofluorescence staining was observed and collected on an Axioskop fluorescence microscope (Zeiss) with the Power Gene 810/Probe and CGH software system (PSI). As unstressed (negative) controls, we used BHK cells stably expressing solely wt- or F508del-CFTR. As stressed (positive) controls, we used the same cells recovering from heat shock (HS) at 43 °C for 90 min (at 0 h and 18 h post-HS).

Statistical analysis

Comparisons between slopes of lines representing degradation rates (regression lines) were made using a Student’s t test. On a regression modelling procedure, the slope is known to follow a t distribution [35–37]. Therefore, slopes of two straight lines can be compared using a t distribution with $n_1 + n_2 - 4$ degrees of freedom, where $n_1$ and $n_2$ are the number of points used on the regression procedure in groups 1 and 2, respectively. Differences are considered as significant for P values < 0.05 [35–37].

RESULTS

Levels of Hsp70 in cells stably or transiently transfected with Hsp70 cDNA

We first tested the levels of Hsp70 expression after transient transfection by immunoblot with a specific Ab in cells collected 24 h post-transfection. Hsp70 was not detected in significant amounts in non-Hsp70-transfected CHO cells stably expressing wt- (Figure 1A, lane 1) or F508del-CFTR (Figure 1B, lane 1) nor in mock-transfected cells (lanes 2 in Figures 1A and 1B). These results reveal that liposome transfection does not induce a stress response, contrary to what happens with other methods of DNA delivery, namely viral ones [38].

Expression of Hsp70 was confirmed 24 h post-transfection with the Hsp70-pcDNA3 construct of cells already stably expressing wt- (Figure 1A, lane 3) or F508del-CFTR (Figure 1B, lane 3). MWC expressing endogenous Hsc70 were assessed in non-stressed CHO cells stably expressing wt- (lane 1) or F508del-CFTR (lane 3) and in the same cells allowed to recover for 18 h at 37 °C after HS (Figure 1C, lane 3). Blots were probed with the anti-Hsp70-specific Ab as indicated. For comparison, in (C) levels of endogenous Hsc70 were assessed in non-stressed CHO cells stably expressing wt- (lane 1) or F508del-CFTR (lane 3) and in the same cells allowed to recover for 18 h at 37 °C after HS (lanes 2 and 4 respectively). BHK cells stably expressing wt-CFTR (D) or F508del-CFTR (E) were co-transfected with pcDNA3 containing Hsp70 cDNA (see the Experimental section). Lane 1, non-transfected cells; lane 2, mock-transfected cells (only pcDNA3); lane 3, cells analysed 24 h post-transfection with Hsp70 cDNA; lane 4, cells allowed to recover for 18 h at 37 °C after HS (43 °C for 90 min). Blots were probed with the anti-Hsp70 specific Ab as indicated. For comparison, in (C) levels of endogenous Hsc70 were assessed in non-stressed CHO cells stably expressing wt- (lane 1) or F508del-CFTR (lane 3) and in the same cells allowed to recover for 18 h at 37 °C after HS (lanes 2 and 4 respectively). BHK cells stably expressing wt-CFTR (D) or F508del-CFTR (E) were co-transfected with pcDNA3 containing Hsp70 cDNA (see the Experimental section). Lane 1, non-transfected cells; lane 2, mock-transfected cells (only pcDNA3); lane 3, cells stably transfected with Hsp70 cDNA. Blots were probed for Hsp70.
Figure 2  Localization of HSF1 in cells stably transfected with Hsp70

Intracellular localization of the transcription factor HSF1 was determined by immunocytochemistry in BHK cells expressing wt- (A, C, E, G and I) or F508del-CFTR (B, D, F, H and J). Cells were non-Hsp70-transfected (A and B), mock-transfected, i.e. only pcDNA3 (C and D), stably transfected with the Hsp70-pcDNA3 construct (E and F), exposed to HS at 43 °C for 90 min (G and H) and exposed to same HS conditions followed by recovery for 18 h at 37 °C (I and J). HSF1 is immunodetected in the nucleus of cells immediately after exposure to HS (arrowheads in G and H) and still after 18 h of recovery at 37 °C (I and J) in both cell types. In all other situations, HSF1 is localized in the cytoplasm (arrows in A–F). Scale bar, 50 μm.

Figure 3  Turnover and processing of wt- and F508del-CFTR under Hsp70 stable overexpression

BHK cells stably expressing (A) wt- or (B) F508del-CFTR were stably transfected with the Hsp70 construct (lanes 1–5) or with the same amount of non-recombinant vector as a mock control (lanes 6–10). At 24 h after being seeded, cells were pulse-labelled and chased as before (Figure 2) for 0 h (lanes 1 and 6), 0.5 h (lanes 2 and 7), 1 h (lanes 3 and 8), 2 h (lanes 4 and 9) and 3 h (lanes 5 and 10). Cells were then lysed and immunoprecipitated with an anti-CFTR Ab (see the Experimental section). After electrophoretic separation and fluorography, immature (band B) and mature (band C) forms of CFTR were quantified by densitometric analysis. The rate of degradation of the core glycosylated form (band B) of wt- (C) and F508del-CFTR (D) was determined as the logarithm of the ratio between P, the amount of band B at time t, and P₀, the amount of band B at the start of the experiment. The rate of conversion of the core-glycosylated form (band B) into the fully glycosylated form (band C) of wt-CFTR was also performed for wt-CFTR (E) and determined as the ratio between the amount of band C at time t and the amount of band B at the start of the experiment (P₀).

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Chaperones and cystic fibrosis protein processing and turnover

Turnover and processing of wt- and F508del-CFTR was studied in these cells stably overexpressing Hsp70 (Figures 3A and 3B, respectively). After fluorogram scanning and densitometric analysis of bands B and C, results were plotted graphically. As the turnover of immature CFTR is exponential, results are represented as the logarithm of the ratio of the amount of band B at a given chase time (P) to the amount (P0) at the beginning of the experiment. The degradation rate of the core-glycosylated form of wt-CFTR (band B) after stable Hsp70 transfection was not significantly altered relative to mock-transfected cells as shown by statistical comparison of the slopes of the two lines (see the Experimental section and Table 1; Figure 3C). The same applies to F508del-CFTR turnover under Hsp70 transfection and control conditions (Figure 3D). We also checked for a possible effect of Hsp70 overexpression at the level of CFTR processing efficiency. This was measured as the percentage of mature form at a given time of chase relative to the total amount of immature CFTR at time 0 (P0). No significant change in the maturation efficiency occurred for wt-CFTR under Hsp70 transfection relative to mock transfection (Figure 3E). Similarly to non-Hsp70-transfected cells, F508del-CFTR did not mature under transient Hsp70 transfection (absence of band C in Figure 3B).

Turnover and processing of wt- and F508del-CFTR was also studied at 24 h post-transfection (transient) with the Hsp70-pcDNA3 construct by pulse-chase experiments followed by immunoprecipitation. Results were equivalent to those shown here after Hsp70 stable transfection (results not shown).

**Turnover and processing of wt- and F508del-CFTR after Hsp70 and Hdj-1/Hsp40 co-transfection**

As the overexpression of Hsp70 produces no effect upon CFTR turnover or processing, we studied these processes under co-expression of its co-chaperone Hdj-1/Hsp40, which has been generally described to stimulate Hsp70 folding activity [26].

<table>
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<tr>
<th>Transfection or treatment</th>
<th>Protein</th>
<th>Significance</th>
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<tr>
<td>Hsp70 transfection</td>
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<td>No</td>
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<tr>
<td>Hsp70 stable transfection</td>
<td>wt-CFTR</td>
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<tr>
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<td>F508del-CFTR</td>
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<tr>
<td>4-PBA</td>
<td>wt-CFTR</td>
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<td>DSG</td>
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**Table 1 Statistical comparison of the slopes of the regression lines corresponding to the degradation rate of immature CFTR**

Statistical significance was taken as P < 0.05; comparisons were with mock-transfected controls.

Lane 3). We estimate that the Hsp70 levels after transfection are about the same as those detected for Hsc70 in non-stressed cells (Figure 1C, lanes 1 and 3). Transfection thus doubled the amount of Hsc70/Hsp70 chaperones. As a positive control, Hsp70 was detected at high levels in the same cells analysed 18 h after recovery at 37 °C from HS for 90 min at 43 °C (Figures 1A and 1B, lanes 4) and Hsc70 expression also increased under the same conditions, but not so dramatically (Figure 1C, lanes 2 and 4).

Liposome transient transfection is commonly described as having a success rate not superior to 40%. Therefore, we also produced cell lines stably expressing Hsp70 in cells already stably expressing wt- or F508del-CFTR, to study the effect of Hsp70 overexpression upon CFTR turnover and processing on a balanced Hsp70/CFTR ratio. BHK cells stably expressing wt- or F508del-CFTR under methotrexate selection were transfected with Hsp70 and transfectants isolated with G418 selection. Expression of Hsp70 in G418-selected clonal cells was confirmed by Western blot (Figures 1D and 1E, lanes 1). As for CHO cells (Figures 1A and 1B), Hsp70 was not detected by Western blot in non-Hsp70-transfected cells (Figures 1D and 1E, lanes 1) nor in stably mock-transfected cells (Figures 1D and 1E, lanes 2).

To confirm that the Hsp70 detected in the stable transfectants resulted from the expression of the recombinant vector and not from stress induction by the transfection itself, intracellular localization of HSF1 was determined by immunocytochemical techniques.

HSF1 is normally present in the cytoplasm but under stress conditions migrates to the nucleus to activate the stress response [34]. It is thus possible to distinguish between non-stressed cells and cells under stress by determining HSF1 localization. HSF1 was localized in the cytoplasm of non-transfected cells, stably expressing wt- (Figure 2A) or F508del-CFTR (Figure 2B), as well as in stably mock- (Figures 2C and 2D) or Hsp70-transfected cells (Figures 2E and 2F). These observations strongly contrast with what was observed for cells analysed immediately after HS (90 min at 43 °C), in which HSF1 was localized almost exclusively in the nucleus (Figures 2G and 2H), indicating that a stress response was triggered. In cells analysed 18 h after recovery at 37 °C post-HS, immunodetection of HSF1 in the nucleus was less intense and this factor was already localized in the cytoplasm (Figures 2I and 2J). Altogether, data in Figure 2 show that stable transfection of Hsp70 into cells does not trigger a stress response, and neither does expressing the F508del-CFTR mutant in high amounts.

![Figure 4 Immunodetection of Hsp70 and Hdj-1/Hsp40 after transient transfection](image-url)
Levels of Hsp70 and Hdj-1/Hsp40 were assessed by immunoblot with specific Abs in cells collected 24 h post-transfection. Hsp70 and Hdj-1/Hsp40 were not detected in non-transfected cells (Figures 4A–4D, lanes 1) nor in mock-transfected cells (Figures 4A–4D, lanes 2), revealing again that transfection itself does not induce either of these chaperones. In co-transfected cells, expression of Hsp70 and Hdj-1/Hsp40 was confirmed (Figures 4A–4D, lanes 3). The levels of Hdj-1 after transfection were found to be equivalent to those of Hdj-2 (its constitutive homologue) in cells under standard conditions (results not shown).

Turnover and processing of wt- and F508del-CFTR was studied under these levels of Hsp70 and Hdj-1/Hsp40 expression (Figures 5A and 5B, respectively). The degradation rate of the core-glycosylated form of wt-CFTR (band B) was significantly decreased (Table 1) relative to mock-transfected cells, as shown by statistical comparison of the slopes of the two lines shown in Figure 5(C). The same analysis revealed that the degradation rate of the immature form (band B) of F508del-CFTR (Figure 5D) was not significantly altered compared with mock-transfected cells.

The observed increase in the stability of the wt-CFTR immature form did not correspond, however, to an increase in the maturation efficiency for wt-CFTR (Figure 5E). As above, no maturation was detected for F508del-CFTR (no band C in Figure 5B).

**Turnover and processing of wt- and F508del-CFTR after treatment with DSG and 4-PBA**

4-PBA was described to facilitate CFTR processing by a mechanism involving down-regulation [28]. For this reason, we studied turnover and processing of wt- and F508del-CFTR after treatment with 5 mM 4-PBA for 48 h (Figures 6A and 6B, respectively). The degradation rate of the core-glycosylated form of wt-CFTR (band B) was not significantly altered relative to mock-transfected cells, as shown by statistical comparison of the slopes of the two lines shown in Figure 6(C) (Table 1). In contrast, analysis of F508del-CFTR revealed that the degradation rate of the immature form (Figure 6D) was significantly increased when compared with non-treated cells (Table 1). However, this destabilization of the F508del-CFTR immature form did not correspond to the detection of the mature form of the mutant protein (no band C in Figure 6B).

Another compound described to facilitate CFTR traffic to the cellular membrane by disrupting interaction with Hsc70/Hsp70 is DSG [29]. After DSG treatment, our results did not show evi-
**Figure 6** Turnover and processing of wt- and F508del-CFTR following 4-PBA treatment

CHO cells stably expressing (A) wt- or (B) F508del-CFTR were treated with 5 mM 4-PBA (lanes 6–10) or non-treated (lanes 1–5). After 48 h treatment, cells were pulse-labelled and chased as before (Figure 2) for 0 h (lanes 1 and 6), 0.5 h (lanes 2 and 7), 1 h (lanes 3 and 8), 2 h (lanes 4 and 9) and 3 h (lanes 5 and 10). Cells were then lysed, immunoprecipitated with an anti-CFTR Ab and analysed as before (see Figure 3) to determine the degradation rate of wt- (C) and F508del-CFTR (D) as well as the rate of conversion of the core-glycosylated form (band B) into the fully glycosylated form (band C) of wt-CFTR (E).

Dence of significant changes (Figure 7) in either the turnover or processing of CFTR (Figures 7A and 7B). A slight, but not significant, stabilization of the immature form of CFTR (band B) was observed (Figure 7B). However, it did not correspond to a detectable increase in the efficiency of processing, as virtually the same amount of band C was observed after 3 h of chase (Figure 7C). A summary of all comparisons made between slopes of lines determined in this study is shown in Table 1.

**DISCUSSION**

**Hsp40 is required for stabilization of CFTR by Hsp70**

We tested here whether overexpressing Hsp70 in vivo would enhance the folding efficiency of CFTR, a transmembrane protein co-translationally inserted into the ER, as previously suggested for Hsc70 by experiments performed in vitro solely with part of the NBD1 of CFTR [24]. Our results show that high levels of Hsp70, induced by transient (results not shown) or stable transfection (Figure 3), do not influence the degradation rate or the efficiency of processing of either wt- or F508-CFTR. The fact that we used human Hsp70 and the above-mentioned in vitro studies used Hsc70 (the cognate homologue) does not seem relevant, as the proteins share 81% identity, and the most striking difference between the two genes is found at the level of genomic organization [39]. Indeed, Hsc70 and Hsp70 are thought to play essentially the same chaperoning role in the cell, although Hsp70 is more drastically induced under stress conditions to cope with the increased amount of unfolded protein in the cell [38].

We also demonstrate here that cells expressing the F508del-CFTR do not have increased levels of Hsp70 in comparison with cells expressing wt-CFTR (lanes 1 in Figures 1A and 1B), and are therefore not under stress (Figure 2B), contrary to what might be expected of cells expressing a misfolded protein in high amounts. This observation alone suggests that the amount of Hsc70 is not limiting in these cells.

We then tested whether overexpression of Hsp70 in conjunction with its co-chaperone Hdj-1/Hsp40, shown to stimulate Hsp70 folding activity [40], would produce an effect on CFTR stability. We show that the immature form (band B) of wt-CFTR, but not of F508del-CFTR, is stabilized under such conditions.

Several studies have described that overexpression of Hsp70 family members leads to an increase in the folding of different cytosolic substrates/reporter proteins [41,42]. Recently, it was demonstrated that in mammalian cells overexpression of Hsp70 alone is sufficient to attenuate luciferase inactivation by HS and to enhance its reactivation [26]. For CFTR, high levels of Hsp70 induced by HS (Figures 1A and 1B, lanes 4) do not cause an
Figure 7 Turnover and processing of wt-CFTR following DSG treatment

CHO cells stably expressing wt-CFTR were treated with 50 μg/ml DSG (lanes 6–10) or non-treated (lanes 1–5). After 72 h treatment, cells were pulse-labelled and chased as before (Figure 2) for 0 h (lanes 1 and 6), 0.5 h (lanes 2 and 7), 1 h (lanes 3 and 8), 2 h (lanes 4 and 9) and 3 h (lanes 5 and 10). Cells were then lysed, immunoprecipitated with an anti-CFTR Ab and analysed as before (see Figure 3) to determine the degradation rate of wt-CFTR (B) as well as the rate of conversion of the core-glycosylated form (band B) into the fully glycosylated form (band C) of wt-CFTR (C).

The novelty here is that such an effect is observed for a transmembrane protein, although most of its domains lie within the cytoplasm. It is also striking that for this polytopic membrane protein the combined effect of both Hdj-1 and Hsp70 is necessary to induce increased stability. In the case of CFTR, and perhaps other inefficiently folding multi-domain proteins, the contribution of the co-chaperone seems to determine the modulation of Hsp70's effect upon CFTR stabilization and/or folding. This is in agreement with previous results showing that endogenous Hdj-2 co-operates with Hsc70 in the biogenesis and folding of wt-CFTR [21].

We did not test here the effect of overexpressing Hdj-1/Hsp40 alone, but it is very unlikely that this would produce any effects on turnover or processing (results not shown). Although negative results should be interpreted with caution, similar repeated experiments following both transient and stable transfections with Hsp70 do produce significant changes in the degradation of CFTR (see Figure 3).

However, increasing the cellular level of Hsp70 may not itself cause an increase in its chaperoning activity, as its effect upon substrate binding/release is influenced by several positive and negative modulator proteins [38]. Therefore, we studied the effect of co-overexpressing Hdj-1/Hsp40 and observed a stabilization of the immature form of wt-CFTR. This observation suggests that Hsp70 needs its Hsp40 co-chaperone to produce an effect on wt-CFTR that is not visible for F508del-CFTR. Similarly, Michels et al. [43] showed that co-transfection of Hdj-1/Hsp40 enhanced the reactivation of heat-denatured firefly luciferase, which had already been observed following Hsp70 transfection alone. Similarly, length-dependent aggregation of polyglutamine is suppressed by the Hsp70 and Hsp40 chaperones [44].

The novelty here is that such an effect is observed for a transmembrane protein, although most of its domains lie within the cytoplasm. It is also striking that for this polytopic membrane protein the combined effect of both Hdj-1 and Hsp70 is necessary to induce increased stability. In the case of CFTR, and perhaps other inefficiently folding multi-domain proteins, the contribution of the co-chaperone seems to determine the modulation of Hsp70's effect upon CFTR stabilization and/or folding. This is in agreement with previous results showing that endogenous Hdj-2 co-operates with Hsc70 in the biogenesis and folding of wt-CFTR [21].

We did not test here the effect of overexpressing Hdj-1/Hsp40 alone, but it is very unlikely that this would produce any changes. Although initially described to have residual intrinsic chaperoning activity [45], Hdj-1/Hsp40 was more recently shown to neither promote folding of soluble substrates such as luciferase [43] nor render cells thermoresistant without Hsp70. These results support the notion that Hdj-1/Hsp40 itself has no chaperone activity [46].

Effects of Hsp70 and Hdj-1/Hsp40 on CFTR retrotranslocation and/or degradation in the ER

Our results show no evidence of changes in the degradation rate of wt- or F508del-CFTR immature forms under overexpression of Hsp70 alone, thus suggesting that this chaperone does not play a critical role in retrograde transport and/or ER degradation in mammalian cells [18]. Alternatively, Hsc70 levels in these cells may already be sufficient to participate in ER-associated degradation.

Recently, the yeast Hsp70 orthologue SSA1 was described to facilitate ER degradation of CFTR heterologously expressed in Saccharomyces cerevisiae. In this study conditional ssa1 mutants were used to diminish the cellular level of this chaperone. It is possible that in mammalian cells there is a greater redundancy of molecular chaperones than in yeast and thus, in this lower eukaryote, the amount of chaperones present may be limiting the rate of retrograde transport and/or ER degradation. Depletion of Hsc70 would be a potent strategy to determine whether the role observed for SSA1 in yeast is also played by Hsc70/Hsp70 in mammalian cells. However, direct attempts to achieve a significant reduction in the cellular levels of Hsc70 (by anti-sense strategy) were unsuccessful (C. M. Farinha, unpublished work), since this chaperone is self-regulated and thus experimentally decreased levels were rapidly restored.
Nevertheless, when Hdj-1/Hsp40 and Hsp70 are co-expressed, the effect observed is a stabilization of the ER immature form of wt-CFTR, i.e. opposite to what was described for yeast [19]. This may be due to intrinsic differences between the ER degradation systems of yeast and mammalian cells. Alternatively, in mammalian cells, Hsp70/Hsc70 could be under more sophisticated regulation than in yeast. Indeed, several co-chaperones, such as Hdj-1 and -2, Hip, C-terminus of Hsc70-interacting protein (CHIP) and Bag-1, some of which have not been described to occur in yeast, were shown to cause different effects on mammalian Hsc70/Hsp70 activity [19,38]. This hypothesis is supported by the fact that, instead of Hdj-1/Hsp40, the Hsp70 co-chaperone CHIP is overexpressed, destabilization of immature CFTR occurs [47]. This is probably due to the recently demonstrated activity of CHIP as an intrinsic E3 ubiquitin ligase that promotes ubiquitylation involving Hsc70, thus stimulating the ability of the latter to deliver substrates to the proteasome [48].

No effect is observed for F508del-CFTR

We show that, in contrast to what is observed for wt-CFTR, increasing the levels of Hsp70 either by transient or stable transfection or even by stimulating its activity through Hdj-1/Hsp40 co-expression has no effect upon F508del-CFTR stability.

The observed differences between wt- and F508del-CFTR are probably due to the distinct cytosolic domain conformations of the two variants. Indeed, using comparative limited proteolysis at steady state, Zhang et al. [9] showed that wt-CFTR assumes two distinct conformations, based on the respective protease susceptibilities: one sensitive (unstable band B) and another resistant (stable band B). Additionally, these authors also showed that the proteolytic pattern of F508del-CFTR is similar to that of immature band B of wt-CFTR but quite different from that of stable wt-CFTR band B or mature band C. It was thus suggested that F508del-CFTR, does not achieve the stable folded conformation [9].

Our results, observing a differential effect on the immature conformations of wt- and F508del-CFTR, upon overexpression of Hsp70 and Hdj-1/Hsp40, strongly favour the latter hypothesis [9]. The Hsp70/Hdj-1 pair thus seem to facilitate, and indeed accelerate, the folding of an intermediate conformation of wt-CFTR, while not acting upon the major off-pathway conformation that occurs for F508del-CFTR.

Impact of overexpression of Hsp70 and Hdj-1/Hsp40 on CFTR processing

We also show that the overexpression of Hsp70 either by itself or in conjunction with Hdj-1/Hsp40 produces no significant effect on the conversion of the immature form of wt-CFTR into its fully glycosylated form. These results suggest that the observed stabilization by the Hsp70/Hdj-1 chaperones occurs at an early stage of CFTR biogenesis, distinct from the events that cause its escape from the ER. The fact that Hsp70/Hdj-1 overexpression does not have an impact upon CFTR maturation (wt or F508del) suggests that lack of these chaperones is not the limiting step in CFTR processing.

Effects of 4-PBA and DSG upon CFTR turnover and processing

A direct effect of 4-PBA in diminishing the interaction of Hsc70 with CFTR has never been shown, although a decrease in Hsc70 cellular levels under 4-PBA treatment was described [28]. However, 4-PBA is known to be an inducer of peroxisome proliferation [49], so we have performed experiments to analyse the levels of Hsc70, catalase (the major peroxisome protein) and actin (as an internal control) following 4-PBA treatment (results not shown). Results strongly favour the hypothesis that the reduction in Hsc70 levels under 4-PBA is not a specific reduction in the absolute levels of this chaperone. Rather, it seems to be an indirect consequence of the marked increase of peroxisomal proteins, thus resulting in an apparent reduction in the relative levels of all other cellular (non-peroxisomal) proteins. Moreover, our results with 4-PBA show that this compound (whether or not specifically inhibiting Hsc70 binding to CFTR) increases the instability of F508del-CFTR.

We cannot exclude the possibility that an effect may be produced by DSG on F508del-CFTR, which may be detected by the very sensitive methodology of patch-clamp determination of CFTR chloride channel function. However, by biochemical methods, we cannot detect any effect of DSG upon CFTR processing, although a modest decrease in the interaction of CFTR with Hsc70 is observed by sequential immunoprecipitation (results not shown).
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