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Rescue of F508del-CFTR by RXR motif inactivation triggers proteome modulation associated with the unfolded protein response

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ABSTRACT

F508del-CFTR, the most common mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, disrupts intracellular trafficking leading to cystic fibrosis (CF). The trafficking defect of F508del-CFTR can be rescued by simultaneous inactivation of its four RXR motifs (4RK). Proteins involved in the F508del-CFTR trafficking defect and/or rescue are therefore potential CF therapeutic targets. We sought to identify these proteins by investigating differential proteome modulation in BHK cells over-expressing wt-CFTR, F508del-CFTR or the revertant F508del/4RK-CFTR. By 2-dimensional electrophoresis-based proteomics and western blot approaches we demonstrated that over-expression of F508del/4RK-CFTR modulates the expression of a large number of proteins, many of which are reported interactors of CFTR and/or 14-3-3 with potential roles in CFTR trafficking. GRP78/BiP, a marker of ER stress and unfolded protein response (UPR), is up-regulated in cells over-expressing either F508del-CFTR or F598del/4RK-CFTR. However, over-expression of F508del/4RK-CFTR induces the up-regulation of many other UPR-associated proteins (e.g. GRP94, PDI, GRP75/mortalin) and, interestingly, the down-regulation of proteasome components associated with CFTR degradation, such as the proteasome activator PA28 (PSME2) and COP9 signalosome (COPS5/CSN5). Moreover, the F508del-CFTR-induced proteostasis imbalance, which involves some heat shock chaperones (e.g. HSP72/Hpa2), ER-EF-hand Ca^{2+}-binding proteins (calumenin) and the proteasome activator PA28 (PSME2), tends to be 'restored', i.e., in BHK cells over-expressing F508del/4RK-CFTR those proteins tend to have expression levels similar to the wild-type ones. These findings indicate that a particular cellular environment orchestrated by the UPR contributes to and/or is compatible with F508del/4RK-CFTR rescue.
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1. Introduction

Cystic fibrosis (CF) is a common autosomal recessive inherited disorder most prevalent in the Caucasian population [1,2]. The disease is caused by mutations in the gene coding for the CF transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel that functions at the apical surface of epithelial cells [3,4]. About 1500 different disease-causing mutations affecting various aspects of CFTR function have been reported for the *CFTR* gene (http://www.genet.sickkids.on.ca/cftr). The predominant CF mutation, found in more than 70% of CF patients in Northern Europe and North America, is the deletion of the phenylalanine at position 508 (F508del) in the first nucleotide binding domain [5]. F508del is the prototypical example of a class-II CF mutation [6–8]. Mutations in this class are responsible for defective intracellular trafficking where the translated immature mutant CFTR proteins are mostly retained in the endoplasmic reticulum (ER) and fail to mature into fully glycosylated forms at the cell membrane [9,10]. The F508del-CFTR trafficking defect is temperature-sensitive, as incubation at the permissive temperature of 27–30 °C results in partial export of the protein from the ER to the cell surface where it functions similarly to the wild-type protein [11]. Several attempts to promote normal processing and function of F508del-CFTR have been described as potential therapeutic strategies in the management of CF [12–14]. Cells expressing F508del-CFTR regain their ability to exhibit forskolin-dependent chloride transport at the cell surface after treatment with a number of chemical/pharmacological chaperones known to stabilize proteins in their native conformation [15–17]. Mutagenesis of arginine-framed tripeptides (AFTs or RXR motifs), reported to be involved in the ER quality control of many membrane proteins, has also been used as a strategy to redirect F508del-CFTR to the cell membrane [18,19]. Simultaneous inactivation of two or of the four CFTR RXR motifs (termed 4RK) by substitution of arginine by lysine at positions R29, R516, R555 and R766 allows nascent F508/4RK-CFTR to escape ER quality control and function at the cell surface [20,21]. Other mutations in *cis* with F508del also have a rescue effect on the misfolded protein [22–24].

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Although the effect of these revertant mutations on defective F508del-CFTR trafficking and function is well documented, the corresponding molecular mechanism is not fully elucidated.

In the present study we investigated by proteomics whether baby hamster kidney (BHK) cells, a popular heterologous model system for examination of CFTR processing and function, underwent differential proteome modulation in response to the expression of wild-type-CFTR, F508del-CFTR or F508del-4RK-CFTR. We hypothesized that proteins differentially expressed in response to processing, trafficking and function of a particular CFTR isoform might be involved in these cellular events. This approach may contribute to a better understanding of the cell quality control response that recognizes the trafficking defect of F508del-CFTR and promotes its partial rescue to the cell surface when the four RXR motifs are inactivated.

Ultimately, this approach could reveal novel targets for CF therapy.

2. Materials and methods

2.1. Cells

Baby hamster kidney (BHK) cell lines not expressing CFTR (BHK-L) or stably expressing wild-type (wt-CFTR), F508del-CFTR or F508del/4RK-CFTR (previously produced in our department by M Roxo-Rosa) were cultivated as described [24]. Cells were trypsinized and washed once in PBS and pellets of 5×10^6 cells were quickly frozen on dry ice and stored at 80 °C until required for analysis.

2.2. 2-Dimensional electrophoresis (2DE) analysis

2DE was performed as previously described [25] with the following modifications. Pellets of 5×10^6 cells were lysed in 450 l lysis buffer [7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 4% (w/v) CHAPS (Sigma-Aldrich), 60 mM dithioerythritol (Sigma-Aldrich), 0.75% (v/v) ampholine 3.5–10.0 (GE Healthcare), 0.25% (v/v) ampholine 4.0–6.0 (GE Healthcare)]. Total extracted proteins were loaded onto 18 cm Immobiline DryStrips (GE Healthcare) with a nonlinear wide range pH gradient (pH 3–10) and isoelectrically focused on an IPGphor IEF system (Amersham Bioscience) for a total of 60 kVh. Second dimension analysis was performed using 8–16% (w/v) gradient polyacrylamide gels run overnight at 3 W/gel (Ettan DALT*twelve* System, GE Healthcare) as described [25]. Protein visualization was carried out by Coomassie "Blue Silver" staining [26].

2.3. 2DE-map analysis

Five or six replica gels per cell line were performed, giving a total of 21 2D gel images. Digitalized images of Coomassie-stained 2DE-gels were obtained using an ImageScanner (GE Healthcare) and analysis was performed using Progenesis PG200v2006 software (Nonlinear). To standardize the intensities of Coomassie staining, analysis was carried out by taking into account the normalized volume of spots (or %Vol, *i.e.*, the volume of each spot over the volume of all spots in the gel). An average gel image was created for each cell line from each group of gels, with a maximum of two absences allowed, meaning that all proteins were present in at least three gels of each group. The difference in expression levels between cells for a given protein was statistically assessed using the ANOVA test, for n observations, where n is the number of cell types (groups) analysed. Differences over 1.5 times were considered statistically significant for $p<0.05$.

2.4. PMF analysis

After spot removal from gels, protein spots were processed as described [27]. Peptides were eluted with 0.8 l of 10 mg/ml -CHCA (alpha-cyano-4-hydroxycinnamic acid) (LaserBio Labs), prepared in 70% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid, and were directly applied to the MALDI plate, using POROS R2 (Applied Biosystems) columns [28]. Sample peptides were assayed for PMF in a Voyager-DE™ STR MALDI-TOF (Applied Biosystems). Spectra were acquired using the Voyager Instrument Control Panel Software v5.10 (Applied Biosystems). Data Explorer v4.4 (Applied Biosystems) was used for spectra processing, m/z (mass-to-charge ratio) peak evaluation was performed by MoverZ (ProteoMetrics, 2001) using S/N of 5. External calibration was performed using peptide calibration pepmix1 (LaserBio Labs).

PeakErazor (v1.76, Lighthouse Data) was used to filter contaminant m/z peaks and when possible to perform internal calibration. Monoisotopic peptide masses were used for protein identification with Mascot (http://www.matrixscience.com/) PMF software (MSDB database — 3,239,079 sequences, 2006.08.31). Trypsin was the digestion enzyme and one missed cleavage site was allowed. A peptide tolerance of 50–100 ppm was used and Cys carbamidomethylation and Met oxidation were set as fixed and variable amino acid modifications, respectively. Proteins were identified by searching the Swissprot database with a taxonomic restriction to mammalian protein sequences (339,491 sequences searched). The criterion used to accept the identification was a significant homology score achieved in Mascot ($p<0.05$) and a minimum of 3 peptides matched.

2.5. Combined MS+MS/MS analysis

The sample preparation protocol for combined MS+MS/MS was very similar to that used in the MS analysis. After trypsin digestion, supernatants were collected and extraction of tryptic peptides was performed as previously described [29]. Samples (0.5 l) were spotted on the MALDI plate and 0.5 l of 5 mg/ml -CHCA was added to the sample.

Peptides were analysed on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF ion optics. Data were acquired in positive MS reflector mode with six spots of standard (Calibration Mixture 2, Applied Biosystem) used for calibration (4000 Series Explorer Software v3.0 RC1). MS spectra were obtained by 1000 shot/subspectrum accumulations. The three precursor peaks with the best S/N ratios were selected from each spectrum for MS/MS analysis. For MS/MS spectra a maximum of 5200 laser shots were accumulated.

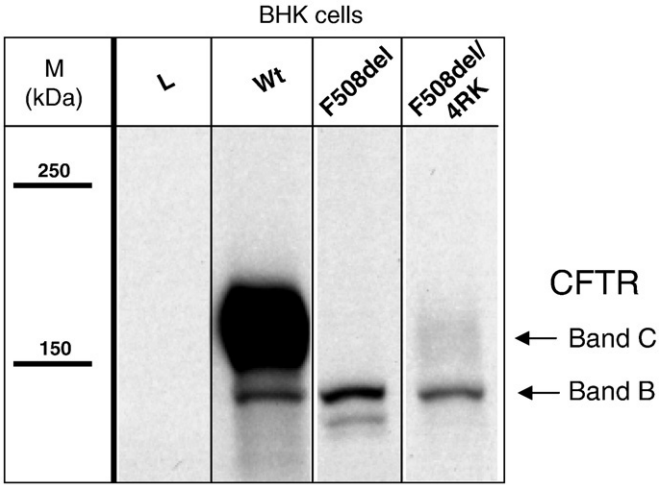


Fig. 1. Western-blotting analysis of BHK cells not expressing (L) or stably expressing wt-CFTR, F508del-CFTR or F508del/4RK-CFTR. Total protein extracts were prepared from cells and resolved (50 µg/lane) on a 7% SDS-polyacrylamide gel before electrophoretic transfer to nitrocellulose for immunodetection of CFTR using M3480 anti-CFTR antibody (1:1000). The presence of the mature fully glycosylated form of CFTR (B and C) is observed only in cells expressing wt- and F508del/4RK-CFTR. F508del-CFTR is expressed mainly in an immature core-glycosylated form (Band B).

Data interpretation was carried out using GPS Explorer software (Version 3.5, Applied Biosystems) and a local copy of the MASCOT search engine (Version 2.0).

For combined MS+MS/MS analysis all peptide mass values were considered monoisotopic, a MS mass tolerance was set at 50 ppm and a MS/MS fragment tolerance was set at 0.25 Da. Trypsin was designated as the digestion enzyme and one missed cleavage site was allowed. Cys carboamidomethylated and Met oxidation were assumed as fixed modification and variable modification, respectively. MS peaks with S/N greater than 5 and MS/MS peaks with S/N greater than 3 were searched against the Swissprot database with a taxonomic restriction to rodent protein sequences (Swissprot 2008.06.09: total 385,721 sequences, of which 24,197 are from rodents). The criterion used to accept the identification was a significant homology score achieved in Mascot (p b 0.05).

2.6. Data analysis

The Gene Ontology Database (http://www.geneontology.org) was used to search for biological processes, cellular components and molecular functions associated with the proteins identified (GO terms) [30]. This made it possible to classify proteins into different functional groups. In order to collect all the available information about each protein, PIKE (Protein Information and Knowledge Extractor) software was used (http://proteo.cnb.uam.es:8080/pike/).

Protein interactions were evaluated through Cytoscape software v2.5.0. Intact was used as the protein–protein interaction database (version 20071020) [31]. Before use, the bioinformatics tools were updated according to the software instructions with the most recent data from the literature on CFTR interactors [32,33], 14-3-3 interactors [34–36] and protein sub-cellular localization [37–39].

2.6. Western blot

Protein extracts from cell lines were quantified (Bradford; Bio-Rad protein assay) and 50 µg or 15 µg of each cell sample were separated in triplicate on 7% or 4–12% (w/v) polyacrylamide gels, transferred to nitrocellulose membranes (Schleicher & Schuell) and analysed by western blot as described [40]. Membranes were probed with 1:1000 mouse anti-CFTR (Chemicon), 1:3000 rabbit anti-GRP78 (Sigma-

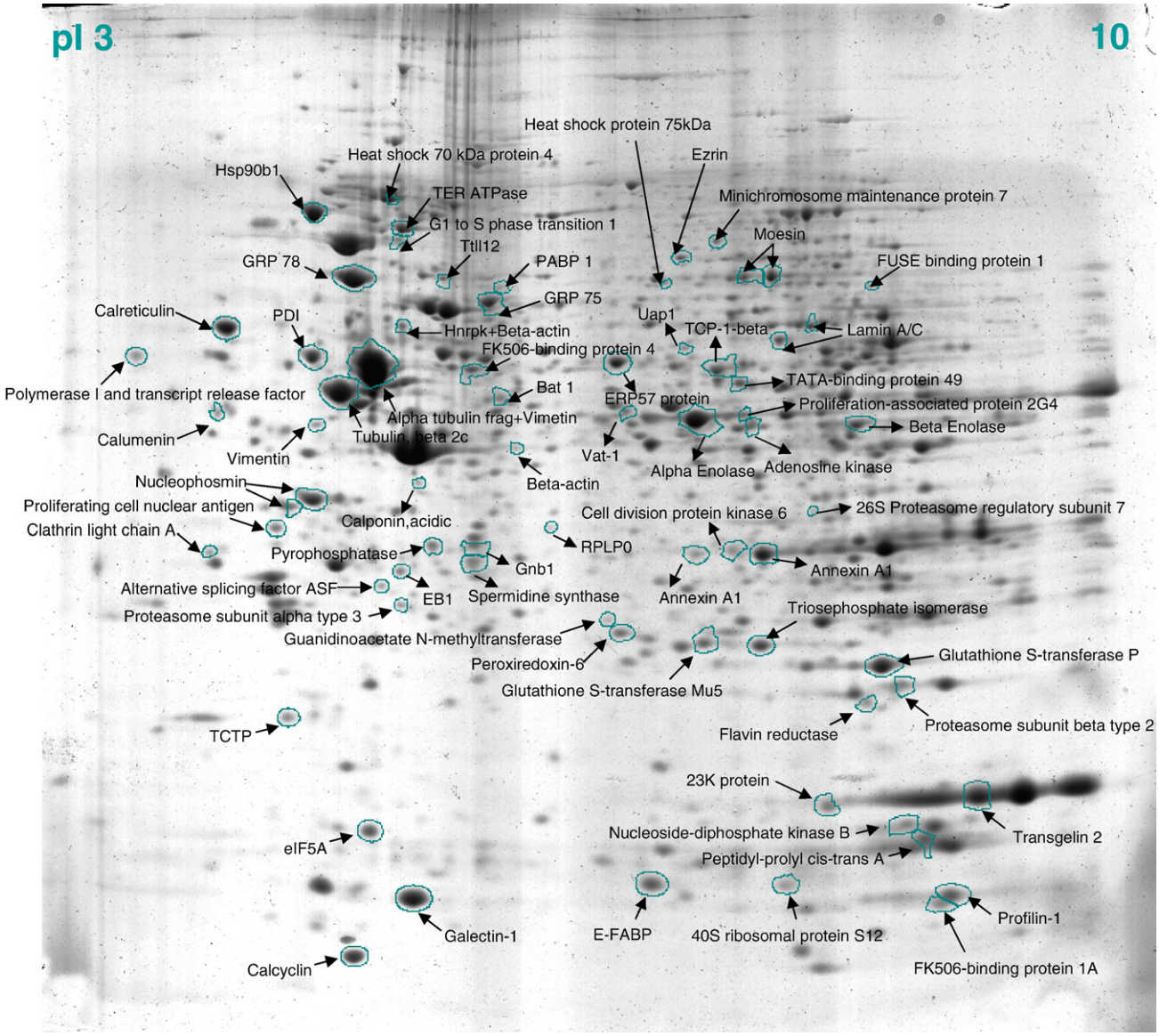


Fig. 2. 2DE reference map of the proteome of BHK-wt, BHK-F508del, BHK-F508del/4RK and BHK-L cell lines. The 71 protein spots identified as differentially expressed between the cell lines are indicated (ANOVA test, p b 0.05; $n = 5/6$ gels/each group, total $n = 21$). These differentially expressed proteins were subsequently identified by mass spectrometry (see Tables 1SD and 2SD of Supplemental data).

Aldrich), 1:500 mouse anti-PDI (Santa Cruz Biotechnology), 1:2000 rabbit anti-GRP94 (Abcam), 1:500 mouse anti-GRP75 (Abcam), 1:1000 rabbit anti-S100A6 (Abcam), 1:1000 mouse anti-HSP72 (Stressgen), 1:1000 goat anti-PSME (Abcam), 1:10,000 mouse anti-COPS5 (Abcam), 1:3000 goat anti-calumenin (Santa Cruz Biotechnology) or 1:500 mouse anti-HSP75 (BD Biosciences), for 2 h at RT and developed using enhanced chemiluminescence-ECL (GE Healthcare). Antibody dilutions were all made in PBS plus 5% fat free milk.

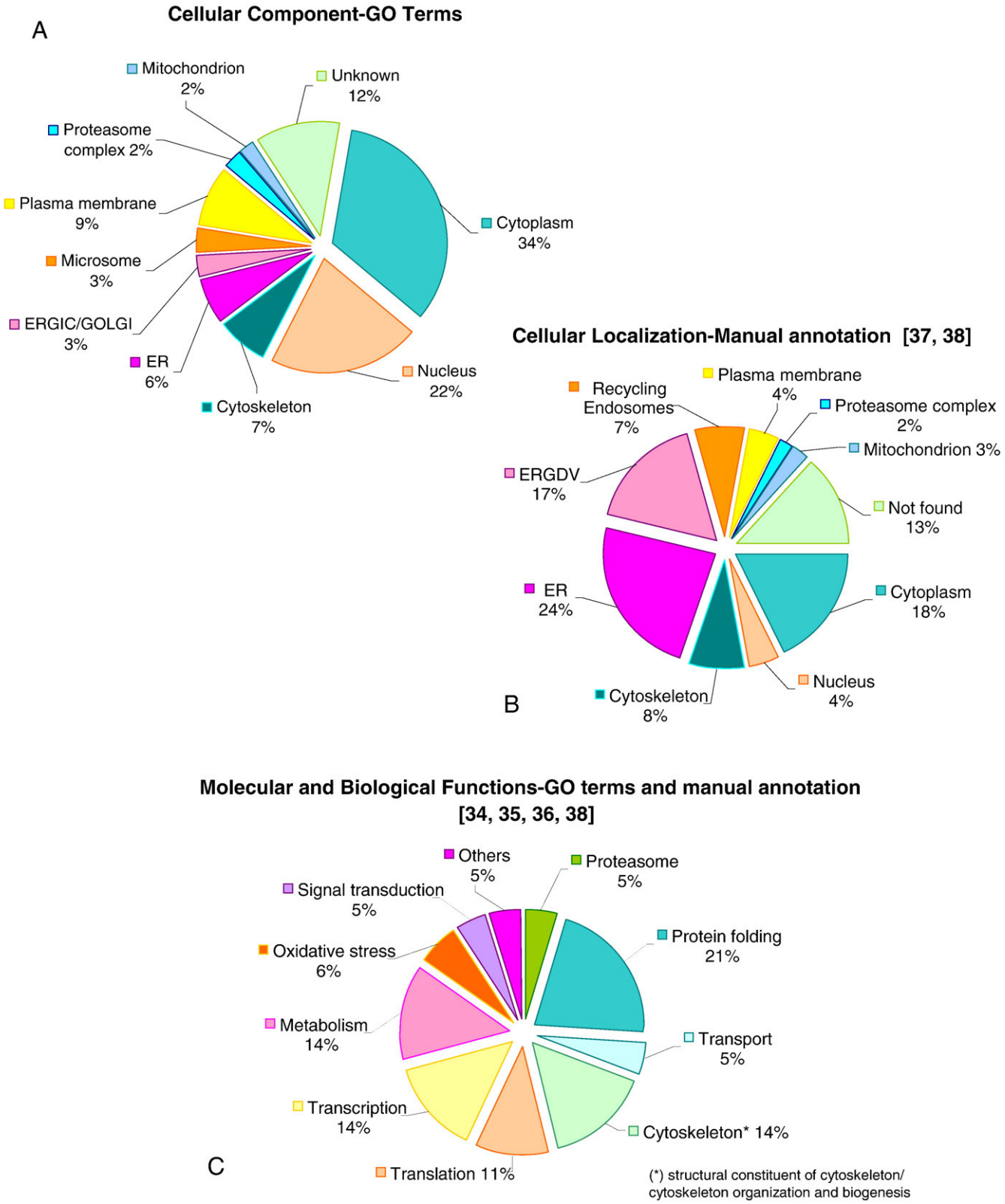
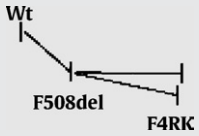
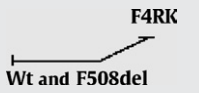
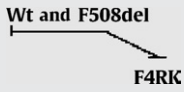


Fig. 3. Graphical representation of cellular component (A), cellular localization (B) and molecular and biological functions (C) of the identified proteins. (A) is based on Gene Ontology (GO) annotation, (B) on manual annotation from recent literature [37,38], and (C) on manual annotation from recent literature [34–36,38] and completed with GO terms when no information was available (see also Table 3SD of Supplemental data).

Table 1
Expression profile behaviours of proteins, including CFTR and 14-3-3 interactors, that are differentially expressed in BHK-wt, BHK-F508del and BHK-F4RK cells.

Group		Expression	Proteins (gene name)
1	A		Calumenin (*CALU*)*, Lamin A/C (*LMNA*)*#, Heat shock protein 75 kDa (*TRAP1*)(*#), Ezrin (*VIL2*)*#, Minichromosome maintenance protein 7 (*MCM7*), Heat shock 70 kDa protein 4 (*HSPA4*)(*)#, Translationally controlled tumor protein (*TCTP*), Adenosine kinase (*ADK*), Far upstream element binding protein 1(*FUBP1*)(#)
	B		Glutathione S-transferase Mu5 (*GSTM5*), Spliceosome RNA helicase Bat1 (*BAT1*), G1 to S phase transition 1 (*GSPT1*), Cell division protein kinase 6 (*CDK6*), Tubulin tyrosine ligase-like family, member 12 (*TTLL12*)
2	C		Triosephosphate isomerase (*TPI1*), 78 kDa glucose-regulated protein (*HSPA5*)*#
	D		23 K protein (*PEBP1*), Glutathione S-transferase P (*GSTP1*), Proteasome subunit alpha type 3 (*PSMA3*)(*), Microtubuleassociated protein RP/EB family member 1 (*MAPRE1*), Acidic ribosomal protein P0 (*RPLP0*)#, Chaperonin containing TCP1, subunit 2 (*CCT2*)(*)#, Lamin A/C (*LMNA*)*#, FK506-binding protein 1A (*FKBP1A*)(*), Nucleoside-diphosphate kinase B (*NME2*)#, Flavin reductase (*BLVRB*), Alternative splicing factor ASF (*SFRS1*), Clathrin light chain A (*CLTA*)*#, Proliferating cell nuclear antigen (*PCNA*), Polymerase I and transcript release factor (*PTRF*), Polyadenylate-binding protein 1 (*PABPC1*)#
3	E		Vimentin (*VIM*)*, TATA-binding protein 49 (*RUVBL1*)*#, Tubulin, beta 2c (*TUBB2C*)(*)#, UDP-N-Acetylhesoamine pyrophosphorylase (*UAP1*), Protein disulfide-isomerase (*PDI*), ERP57 protein (*ERP57*)*#, Calcyclin (*S100A6*)*#, Moesin (*MSN*), Calreticulin (*CALR*)#, 75 kDa glucose regulated protein (*HSPA9*)*#, Transitional endoplasmic reticulum ATPase (*VCP*)*#, Moesin (*MSN*), Endoplasmic reticulum protein 99 precursor (*HSP90B1*)*#, Alpha enolase (*ENO1*)#, Synaptic vesicle membrane protein V (*VAT1*)#, Proliferation-associated protein 2G4 (*PA2G4*), Beta enolase (*ENO3*)#
	F		Proteasome subunit beta type 2 (*PSMB2*)(*), Profilin-1 (*PFN1*)#, Fatty acid-binding protein (*FABP5*), Eukaryotic translation initiation factor 5A (EIF5A)(#), Peroxiredoxin-6 (*PRDX6*)#, Spermidine synthase (*SRM*), Nucleophosmin (*NPM1*)#, 26S Proteasome regulatory subunit 7 (*PSMD7*)*(#), Transgelin 2 (*TAGLN2*), Guanidinoacetate N-methyltransferase (*GAMT*), Guanine nucleotide binding protein, beta 1 (*GNB1*)(*), Annexin A1 (*ANXA1*)#, FK506-binding protein 4 (*FKBP4*)(*)#, 40S ribosomal protein S12 (*RPS12*), Peptidyl-prolyl cis-trans A (*PPIA*), Beta-actin (*ACTB*)#

CFTR interactors; #14-3-3 interactors; (/#)proteins in the same family as the identified protein are reported in the literature to interact with CFTR/14-3-3, respectively [32–34].

For western blot normalization, all membranes were washed 5 times for 10 minutes with stripping buffer [1.5% (w/v) Glycine, 0.1% SDS (w/v); 1% Tween 20 (v/v); pH 2.2], once with PBS and once with PBS-T. The membranes were then probed as above with mouse anti-tubulin (1:10,000) (Sigma-Aldrich) and developed using ECL. The abundance of the tested proteins in BHK cell lines was calculated from densitometry of immunoblots ($n=3$ replicates) using Progenesis PG200v2006 software.

3. Results

3.1. Proteome modulation in CFTR-expressing BHK cell lines

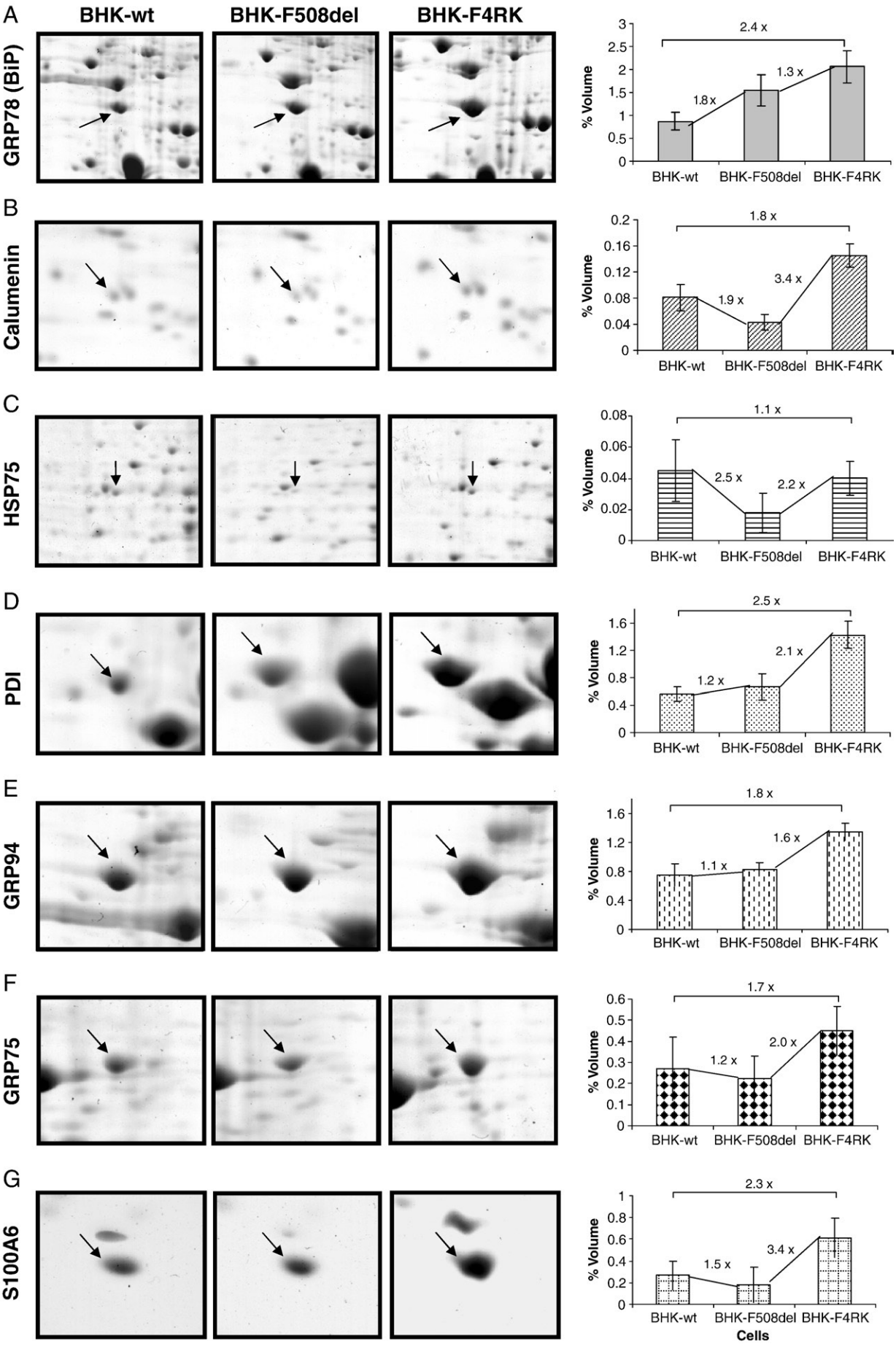
We analysed CFTR-expressing BHK cell lines by 1D-PAGE followed by western blot and confirmed previous findings that in contrast to wt-CFTR, F508del-CFTR is predominantly expressed as a core-glycosylated precursor (Band B, ER form). However, when F508del-CFTR has its four RXR motifs simultaneously inactivated (F508del/4RK-CFTR), a higher molecular weight and fully mature glycosylated product (Band C, plasma membrane form) is readily apparent (Fig. 1).

We next investigated whether BHK cells underwent differential proteome modulation as a consequence of over-expression of different forms of CFTR, and in particular, of the revertant F508del/4RK-CFTR, which is rescued to the cell surface. Using a 2DE-based approach, we analysed and compared the proteomes of non-transfected BHK cell lines (BHK-L) or BHK cell lines stably expressing wild-type CFTR (called here BHK-wt), F508del-CFTR (BHK-F508del) or F508del/4RK-CFTR (BHK-F4RK). Total protein extracts from 5×10^6 cells obtained from each cell line were resolved by 2DE and the 2DE maps were visualized by 'blue silver' Coomassie staining. A total of 21 2D gels (5/6 replica gels per cell line) were obtained and the respective 2DE maps were computer-analysed (see Materials and methods).

By comparing the 2DE maps of BHK-wt, BHK-F508del or BHK-F4RK with BHK-L (non-transfected), a total of 29, 35, and 46 protein spots respectively were found to be differentially expressed (normalized volume differences above 1.5 fold with p (ANOVA) b 0.05) (Table 1SD, Supplemental data). Although the type of regulation and the level of expression were different among these modulated protein spots, eleven of them were differentially expressed in all transfected cells. This cellular response could be a consequence of either the transfection procedure or the over-expression of CFTR, since this protein is not normally expressed by these cells. The majority of the protein spots, however, exhibited specific differential expression in only one or two groups (Table 1SD, Supplemental data), suggesting that this modulation is most likely a response to the type of CFTR expressed. The highest differential proteome modulation was observed in cells expressing the reverted CFTR isoform, F508del/4RK-CFTR.

In total, 71 protein spots were selected for MS analysis using peptide mass fingerprinting (PMF) (52 proteins spots) or MS+MS/MS analysis (19 proteins spots) (Fig. 2 and Tables 2.1 and 2.2SD, Supplemental data). In PMF analysis, when MS spectra allowed identification of two or more orthologous proteins with nearly the same probability and similar MW, only the rodent protein with the best score is presented in this paper (Table 2.3SD, Supplemental data). The MW and the pI localization on the 2D gel for the majority of identified proteins are consistent. In some cases, (e.g. nucleophosmin,

Fig. 4. GRP78/BiP (A), calumenin (B), HSP75/TRAP1 (C), PDI (D), GRP94 (E), GRP75 (F) and S100A6 (G) gel spot representations in BHK-wt, -F508del and -F4RK 2D-maps. The %Volume of these protein spots in each cell line is graphically presented. The fold change between the 3 groups is also given.


A
BHK-wt
BHK-F508del
BHK-F4RK
GRP78 (BiP)
% Volume
2.4 x
1.8 x
1.3 x
BHK-wt
BHK-F508del
BHK-F4RK
B
Calumenin
1.8 x
1.9 x
3.4 x
C
HSP75
1.1 x
2.5 x
2.2 x
D
PDI
2.5 x
1.2 x
2.1 x
E
GRP94
1.8 x
1.1 x
1.6 x
F
GRP75
1.7 x
1.2 x
2.0 x
G
S100A6
2.3 x
1.5 x
3.4 x
Cells

lamin A/C and annexin A1) the same protein was identified in different spots across the 2D gel suggesting the presence of post-translational modifications or truncations in these proteins. The MS identification of 71 differentially expressed spots yielded a total of 65 unique proteins.

Cellular localization, functional annotation and literature searches for these 65 proteins revealed that about 50% of them reside in the ER, ER/COPI, ERGIC (ER-Golgi intermediate compartment), Golgi or TGN (trans-Golgi network) vesicles. Cytoplasmic proteins are also very abundant, including cytoskeleton and proteasome complex constituents (10%) (Fig. 3). Most of the 65 proteins are (i) ER and cytoplasmic molecular chaperones and folding factors, (ii) proteins related to the proteasome complex and protein ubiquitination, (iii) proteins involved in redox regulation of the cell and (iv) proteins involved in cytoskeleton organization (Fig. 3).

3.2. CFTR interactors

Comparing our list of identified proteins with recently published data concerning the CFTR interactome [32,33], we verified that 23 proteins from the list (35%) are the same as, or belong to the same family as, previously reported CFTR interactors (Table 1) [32,33,41]. About half of these CFTR interactors are differentially expressed in BHK-F508del (Table 1: 1A, 1B, 2C, and 2D and Fig. 4). In BHK-F4RK, 9 of the modulated proteins are CFTR interactors (Table 1, 3E, and 3F).

The 23 CFTR interactors are members of the heat shock protein and foldase families, and are described as having prolonged or preferential interaction with F508del-CFTR [13,32]. They include proteasomal subunit proteins (PSMA3, PSMB2, PSMD7) and several CFTR-associated vesicle transport proteins and/or cellular organization proteins (ezrin, vimentin, clathrin light chain A, tubulin beta2, annexin A1).

3.3. 14-3-3 interactors

14-3-3 proteins have been described as mediating forward transport of membrane proteins from the ER to the cell surface through the recognition of RXR motifs [34–36]. Given that no 14-3-3 family members were identified under our study conditions, we searched in our list for 14-3-3 interactor(s) instead. We found 29 proteins reported as 14-3-3 interactors (4 are isotypes/isoforms of previously described interactors) [34–36] corresponding to about 45% of all proteins identified (Table 1). Interestingly, about 50% of these identified 14-3-3 interactors are also CFTR interactors and some are located in the ER/COPI or ER/TGN/ERGIC. Sixteen of these 14-3-3 interactors (55%) are down- or up-regulated exclusively in BHK-F4RK (Table 1, 3E and F). Moreover, in comparison with BHK-F508del, eight 14-3-3 interactors revert their expression in BHK-F4RK to wild-type levels (Table 1, 1A and 1B). Six of them are also CFTR interactors (HSP75, HSPA74, FKBP52, lamin A/C, calcyclin and ezrin).

3.4. Protein expression pro le among the different CFTR-expressing cells

We hypothesized that any protein that is down- or up-regulated in BHK-F508del compared to BHK-wt may be associated with defects in CFTR trafficking and function. If in reverted BHK-F4RK cells the expression level of these proteins is rescued to that observed in BHK-wt cells, or in contrast, the change in expression is more exacerbated in comparison with both BHK-wt and BHK-F508del, we can assume that these proteins have a high probability of being involved in the trafficking and functional restoration of F508del-CFTR achieved by RXR-mutagenic repair.

Table 1 shows a set of proteins (groups 1A and 1B) whose expression levels in BHK-F508del are 1.5 fold different in magnitude (p b 0.05) in comparison to BHK-wt or BHK-F4RK cells. In BHK-F4RK the modulation of these proteins is always away from BHK-F508del levels and towards BHK-wt levels (up- or down-regulated at least 1.3 fold; p b 0.05). Examples in these groups are the CFTR interactors HSP75, HSP74/Hspa4, calumenin, ezrin and FKBP52. All of them are also described as 14-3-3 interactors [34–36,40]. Another protein relevant to CF, annexin I (a 14-3-3 interactor) [27,42], also shows this expression profile.

In contrast, another set of proteins was up- or down-regulated in BHK-F4RK with similar or more pronounced levels of differentiation as those observed in BHK-F508del compared to BHK-wt (Table 1, groups 2C and 2D). These included GRP78/BiP, FKBP12, chaperonin containing TCP1 and the proteasome subunit PSMA3 (all CFTR interactors) [32,33]. Additionally, there is a set of proteins with significantly altered expression only in cells expressing the revertant F508del/4RK-CFTR (Table 1, group 3E and 3F). Some of these proteins are associated with ER stress or the unfolded protein response (UPR).

GRP78/BiP, a hallmark of the UPR [43], was found to be up-regulated in BHK-F508del but also in BHK-F4RK cells (p b 0.05) compared to BHK-wt (Fig. 4A). No up-regulation of GRP78/BiP was observed in BHK-wt compared to BHK-L (Table 1SD, Supplemental data). Besides GRP78/BiP, the ER stress markers GRP94/ERp99 and GRP75/mortalin, and the foldases calreticulin, ERp57 and PDI were also found by 2DE analysis to be exclusively up-regulated in BHK-F4RK cells. In contrast, the proteasome components PSMB2 and PSMD7, which are expressed at similar levels in both BHK-wt and BHK-F508del cells, are down-regulated (p b 0.05) in revertant BHK-F4RK cells (Table 1, 3F and 2D).

3.5. Differential expression evaluated by western blot analysis

Biochemical validation of the 2DE-based proteomic results was performed by western blot analysis (WB) for seven proteins: GRP78/BiP, PDI, GRP94/ERp99, GRP75/mortalin, S100A6, calumenin and HSP75/Trap1. Additionally, although not included in the list of differentially expressed proteins identified by 2DE, three other proteins, the heat shock 70 family member HSP72/Hspa2, the proteasome activator complex subunit 2 (PSME2) and the COP9 signalosome complex subunit 5 (COPS5/CSN5), for which there were antibodies available in the laboratory, were also evaluated by WB to better confirm the cellular pathways believed to be altered under the conditions studied. Alpha-tubulin was examined in parallel as an internal housekeeping control. Protein expression levels were quantified in three independent experiments by densitometric analysis of immunoblots. Although the expression differences were not exactly the same as those found by 2DE analysis, most likely due to sensitivity differences between these two techniques, the validation by WB (Fig. 5) ultimately confirmed the 2DE data.

Several proteins involved in the UPR and/or stress response [44–46] are highly up-regulated in BHK-F4RK cells in comparison with both BHK-F508del and BHK-wt cells (Figs. 5A–F) confirming that UPR is much more evident in these cells. WB experiments also corroborate the 2DE results showing that some proteins like calumenin, HSP75/Trap1 and some members of the Hsp70 family (HSP72/Hspa2) tend to 'revert' their F508del-CFTR-induced expression imbalance in BHK-F508del/4RK cells (Figs. 5F–H). Also, differential modulation of some proteasome components in the different cell lines is confirmed by WB using antibodies against PSME2 and CPOS5/CSN5 (Fig . 5I and J). The up-regulation of PSME2 in BHK-F508del cells is 'reverted' in BHK-F4RK cells (Fig. 5I) while COPS5/CSN5 is exclusively down-regulated in the 'revertant' cells (Fig. 5J).

4. Discussion

In this study we sought to improve our understanding of the repertoire of proteins involved in defective intracellular trafficking of F508del-CFTR. We used CFTR-expressing BHK cell lines to investigate proteome modulation when the F508del-CFTR trafficking defect is partially rescued by mutagenic repair, specifically by inactivation of four CFTR RXR motifs (4RK).

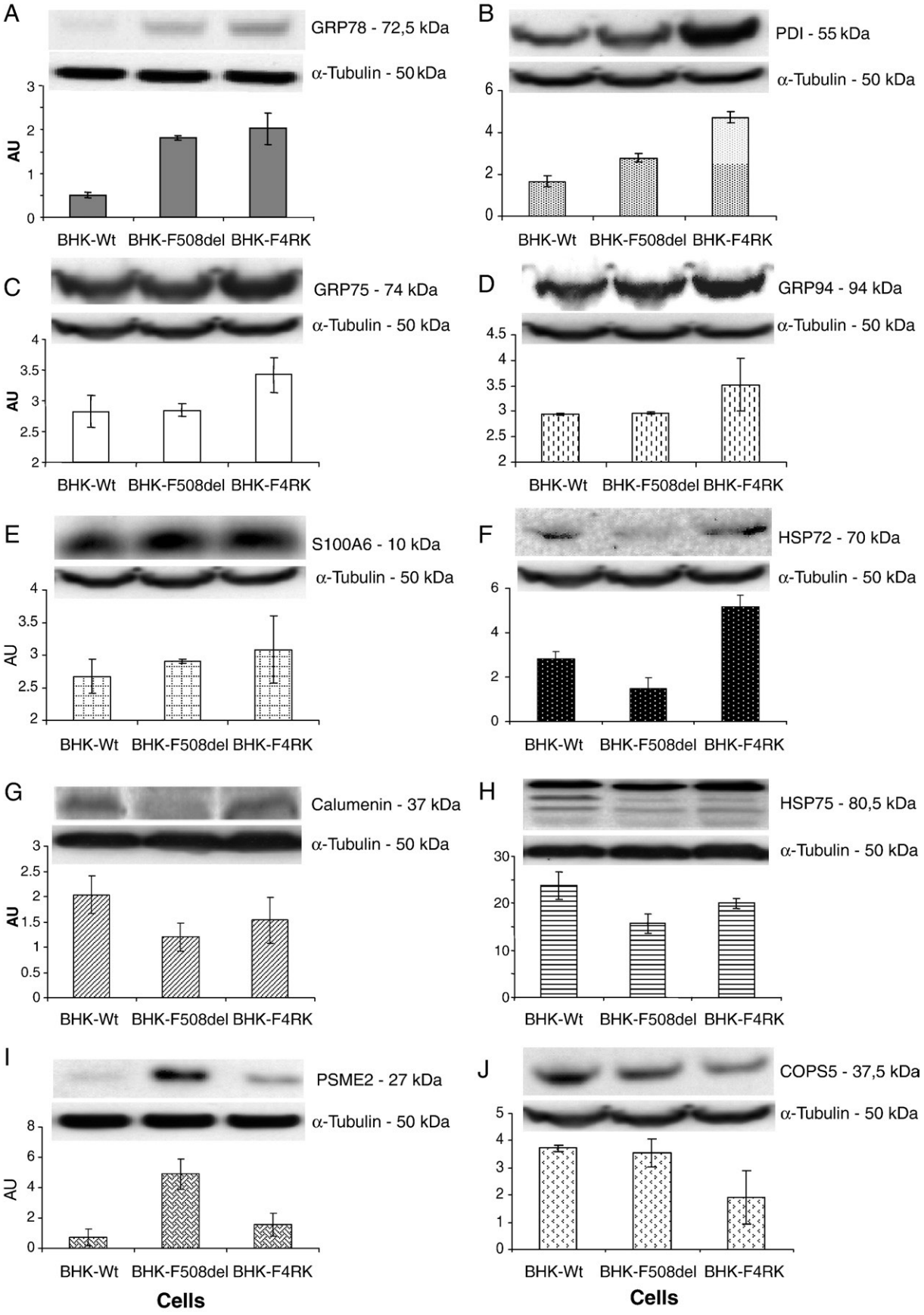


Fig. 5. Western blot validation for GRP78 (A), PDI (B), GRP75 (C), GRP94 (D), S100A6 (E), HSP72 (F), calumenin (G) HSP75 (H), PSME (I) and COPS5 (J). -tubulin was used to normalize the reaction. The antigen–antibody complex was detected by ECL (GE Healthcare). The graphics show the relative normalized abundance of the proteins in BHK cell lines calculated from densitometry of immunoblots ($n = 3$ replicates/each Ab reaction) using Progenesis PG200v2006 software (Nonlinear).

4.1. The 'repaired' form of F508del-CFTR causes ER stress/UPR

In our BHK model system, the over-expression of F508del-CFTR induces up-regulation of the UPR marker GRP78/BiP [44–46], confirming previous findings that UPR is triggered by over-expression of F508del-CFTR but not of wild-type CFTR [47,48]. However, the consequences of over-expressing F508del/4RK-CFTR are much more striking. In BHK-F4RK cells, GRP78/BiP is up-regulated, but so too are several other proteins including ER stress markers (e.g. GRP94/ERp99 and GRP75/mortalin) and foldases (e.g. PDI) (Figs. 4 and 5). These data are consistent with the idea that mutagenic repair through RXR inactivation does not correct the F508del-CFTR folding defect [24], but rather seems to add extra mutations to the protein that are able to induce expression modulation in a larger number of UPR-associated proteins. How this allows the mutant protein to (partially) escape RXR-mediated ER retention and/or retrieval remains to be elucidated.

Several proteins identified in this work have been reported to be direct or indirect CFTR interactors [32,33]. Many of them (e.g. GRP78/BiP, GRP94, HSP72/Hspa2) are exactly the same as those identified by Singh et al. [33] as being up-regulated by either chemical restoration or genetic repair of a CF cell line model (IB3-1) [32,33]. In fact, several approaches that rescue F508del-CFTR from ER-associated degradation (ERAD) and transport it to the cell surface, such as low temperature (our recent findings) [49] and chemical chaperone treatments, cause UPR activation in cells [50–53]. In Calu-3 cells over-expressing recombinant F508del-CFTR, the acute increase in UPR activity induced by incubation with chemical chaperones is accompanied by abolished endogenous CFTR expression, raising some concerns about the positive impact of UPR [48]. Kerbiriou et al. (2007) demonstrated that decreased expression of the UPR-induced transducer ATF6, but not GRP78/BiP, rescued functional membrane localization of F508del-CFTR in the A549 cell line [47]. Further studies will be needed to understand how the modulation of UPR-associated proteins affects F508del-CFTR rescue.

Perturbation of ER folding or processing capacity as a consequence of overloading with unfolded proteins induces the UPR to improve the folding capacity of the ER and re-establish ER homeostasis. The UPR is also known to be intimately linked to ERAD pathways, which are needed to decrease the burden of misfolded proteins in the ER [45]. Recent data suggest however that ERAD could be inhibited by physiologically relevant forms of stress to increase the functional pools of a protein on the cell surface [54]. In revertant BHK-F4RK cells, we observed down-regulation of some proteasome subunits associated with CFTR degradation such as the proteasome activator 28 subunit beta (PA28/PSME2) and a component of the COP9 signalosome (COPS5/CSN5), an alternative cap of the proteasome with high homology to the 19S proteasome cap. Alternative proteasome populations, including the singly and doubly PA28-capped, hybrid and 26S proteasomes, were shown to interact with cytosolic CFTR degradation intermediates [55]. Over-expression of COPS5/CSN5 causes a 25% decrease in F508del-CFTR while its down-regulation decreases the CFTR degradation rate without enhancing cell surface expression [56]. The down-regulation of both PSME2 and COPS5/CSN5, either when using the mutagenic repair strategy (as demonstrated in this study) or low temperature treatments (recently reported by us [49]) to (partially) rescue F508del-CFTR to the cell surface, strongly suggests a significant role for alternate proteasome regulatory caps in the cellular fate of F508del-CFTR.

4.2. Over-expression of 'reverted' F508del/4RK-CFTR is associated with 'restored' expression of some proteins

Interestingly, besides PSME2 (described above), other proteins that were down- or up-regulated in BHK-F508del cells tended to 'revert' to wild-type expression levels when the revertant F508del/4RK-CFTR was expressed (Table 1). These proteins are members of the heat shock family (e.g. HSP72/Hspa2 and HSP75/Trap1) and the ER-EF-hand Ca^{2+}-binding family (e.g. calumenin). Calumenin has been associated with Ca^{2+}-dependent folding and maturation of secretory proteins in the ER lumen throughout the secretory pathway [57,58]. Wang et al. found preferential interaction of this protein with wild-type CFTR [32]. Rescued expression of calumenin in BHK-F4RK gives additional information related to the late events involved in CFTR maturation and trafficking to the cell surface.

The readjustments of F508del-CFTR-induced proteostasis imbalance by rescue strategies such as mutagenic repair (this work) or low temperature treatment (our previous study) [49] also seems to be important for endowing the cell with the ability to (partially) rescue F508del-CFTR to the cell surface.

5. Concluding remarks

In comparison with F508del-CFTR, over-expression of mutagenically 'reverted' F508del/4RK-CFTR activates a UPR/ER stress response involving a large number of proteins that are associated with down-regulation of some proteasome machinery components involved in CFTR degradation (COP9 signalosome and proteasome regulatory cap PA28). This particular UPR/ER stress pathway might be able to generate a favourable cellular environment for (partial) F508del/4RK-CFTR rescue. Most proteins involved are known CFTR and/or 14-3-3 interactors suggesting a potential and relevant role of these proteins in CFTR trafficking. Furthermore, the proteostasis imbalance induced by over-expression of F508del-CFTR is more extreme than that in cells over-expressing the revertant F508del/4RK-CFTR, suggesting that readjustment of protein expression to wild-type levels may be also important for the rescue of F508del-CFTR.

Further studies to understand the interplay of all these proteins and cellular pathways in F508del-CFTR trafficking will provide significant new insights into possible therapeutic targets for the treatment of CF patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbapap.2009.12.013.

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