

Low temperature restoring effect on F508del-CFTR misprocessing: A proteomic approach

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ABSTRACT

To gain insight into the proteins potentially involved in the low temperature-induced F508del-CFTR rescue process, we have explored by two-dimensional electrophoresis (2DE) the proteome of BHK cell lines expressing wt or F508del-CFTR, grown at 37 °C or 26 °C/24 h or 26 °C/48 h followed by 3 h of metabolic labelling with [35 S]-methionine. A set of 139 protein spots (yielding 125 mass spectrometry identifications) was identified as differentially expressed (*p* ANOVA<0.05) among the six phenotypic groups analysed. The data analysis suggests that the unfolded protein response (UPR) induction and some cell-metabolism repression are the major cold-shock responses that may generate a favourable cellular environment to promote F508del-CFTR rescue.

Down-regulation of proteasome regulatory PA28 and/or COP9 signalosome subunit, both involved in CFTR degradation, could also be a relevant cold-shock-induced condition for F508de-CFTR rescue. Moreover, cold-shock may promote the reestablishment of some proteostasis imbalance associated with over-expression of F508del-CFTR. In BHK-F508del cells, the deregulation of RACK1, a protein described to be important for stable expression of CFTR in the plasma membrane, is partially repaired after low temperature treatment.

Together these findings give new insights about F508del-CFTR rescue by low temperature treatment and the proteins involved could ultimately constitute potential therapeutic targets in CF disease.

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1. Introduction

Cystic fibrosis (CF) is an autossomal recessive disorder resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (http://www.genet.sickkids.on.ca/cftr) coding for a membrane cAMP-regulated chloride (Cl⁻) channel that is functional in the apical surface of epithelial cells. F508del (deletion of a phenylalanine residue at position 508) is the most prevalent disease-causing mutation, found in ~70% of the CF chromosomes worldwide [1]. This mutation is the prototype example of class II mutations (defective intracellular trafficking) as the synthesised CFTR is unable to correctly fold and consequently is mostly retained in the ER via the action of molecular chaperones and is degraded [2], likely via ubiquitin/proteasome-dependent pathway [3]. As F508del-CFTR fails to mature and traffic to cell membrane cells expressing this mutant are unable to transport Cl⁻ in response to increases in intracellular cAMP levels.

The folding defect of F508del-CFTR mutant is temperature sensitive [4] and therefore, the most common strategy used to rescue F508del-CFTR to the cell surface is by incubating cells at sub-physiological temperatures (26–30 $^{\circ}$ C).

Given that F508del-CFTR can function as a cAMP-regulated Cl^- channel once it reaches the cell membrane, the

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understanding of the mechanisms that some treatments correct protein folding and trafficking defect of F508del-CFTR could be crucial for the development of an effective therapeutic strategy for CF [5–7].

Low temperature apparently stabilizes the mutant protein during folding in the ER, allowing some CFTR molecules to escape ER quality control and traffic through the Golgi apparatus to the plasma membrane [4]. Very recently, Rennolds et al. [8] suggested that at low temperature F508del-CFTR may use a nonconventional trafficking pathway and by-passes the Golgi apparatus where CFTR becomes Endo-H resistant. Others concluded that at least one rate limiting step in CFTR maturation is its interaction with one or more members of the molecular chaperone family [9]. Although some explanations have been proposed, the mechanisms involved in the restoration of F508del-CFTR trafficking defect by low temperature remain to be elucidated.

We hypothesise that proteins differentially expressed in response to low temperature treatment may be involved in the trafficking rescue of F508del CFTR. To identify such proteins we have investigated and compared by a proteomics approach the protein expression profile of BHK cell lines stably expressing wt- or F508del-CFTR at normal physiologic growth conditions (37 °C) and/or under effect of low temperature (26 °C) incubation for 24 h or 48 h. Our ultimate goal was to propose new targets for development of novel therapeutic strategies for CF.

2. Materials and methods

2.1. Cells and metabolic labelling

BHK cells stably expressing human wild type (wt)- or F508del-CFTR (kindly provided by M Roxo-Rosa, INSA, Lisboa) were cultivated as previously described [10]. Before analysis, 6×10^5 cells were seeded per 35 mm culture dishes (Nunc), grown to about 80% of confluence and then incubated at 37 °C (control) or 26 °C for 24 h or 48 h in the same media without MTX. Cells were incubated 30 min in methionine-free -minimal essential medium (MEM, Gibco, Invitrogen) prior to the metabolic labelling in the same medium with 140 mCi/ml of [³⁵S]methionine (MP Biomedicals), in the last 3 h of incubation at 37 °C or 26 °C, respectively.

2.2. 2DE analysis

Total protein extracts of BHK cells were analysed by twodimensional electrophoresis (2DE) according to [11]. PBS washed cells were lysed directly in IEF lysis buffer [7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 4% (w/v) 3-[(3cholamidopropyl) dimethylamonio]-1-propanesulfonate (CHAPS) (Sigma-Aldrich), 60 mM 1,4-Dithioerythritol (DTE) (Sigma-Aldrich), 0.75% (w/v) ampholine 3.5–10.0 (GE Healthcare), 0.25% 4.0–6.0 (GE Healthcare)]. Supernatants containing the total extracted proteins $(0.8 \times 10^6 \text{ cells/gel})$ were applied onto 18 cm nonlinear pH 3–10 immobiline dry strips (GE Healthcare). After IEF, the strips were applied to 8–16% (w/v) SDS gradient polyacrylamide gels. The proteins on the gel were visualised by autoradiography [12]. Preparative gels were also run with the same conditions as described for analytical gels. The 2D-maps of total protein extract of preparative gels $(5 \times 10^6 \text{ cells/gel})$ were visualised by 'blue silver' coomassie staining as previously described [13], for a consequent compatible MS analysis.

2.3. 2DE gel autoradiography image analysis

The digitalized images (four or five replica gels per condition in a total of 26 gels) were obtained using the ImageScanner (GE Healthcare) and the respective analysis was performed using the Progenesis PG200v2006 (Nonlinear). An average gel image was created for each group of gels, and a maximum of two absences was allowed, meaning that all protein spots were present in at least half of the gels of each group. In order to standardize the intensities of [35S]-methionine radiolabelling among spots present in the several 2DE-maps, 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). The difference in expression levels between groups for a given protein was statistically assessed by using the ANOVA test, for n observations, where n is the number conditions (groups) analysed. Differences over 1.5 fold were considered statistically significant when p < 0.05. Proteins detected/matched in at least two groups were considered for identification.

2.4. MS analysis

After spot excision from gels, protein spots were distained and digested overnight with trypsin (6.7 ng/ l). For peptide mass fingerprinting (PMF) identification, the peptides were eluted with 0.8 l of 10 mg/ml -CHCA (alpha-cyano-4-hydroxycinnamic acid) (LaserBio Labs), prepared in 70% ACN (v/v) and 0.1% (v/v) TFA, directly on top of the MALDI plate, using POROS R2 (Applied Biosystems) columns [14]. PMF was performed in a Voyager-DETM STR MALDI-TOF mass spectrometer (Applied Biosystems). Spectra were acquired using the Voyager Instrument Control Panel Software, version 5.10 (Applied Biosystems). Data Explorer (Applied Biosystems) version 4.4 was used for spectra processing, m/z (mass-to-charge ratio) peak evaluation was performed by MoverZ (ProteoMetrics, New York, USA, 2001) with a S/N of 5. External calibration was performed using peptide calibration pepmix1 (LaserBio Labs). PeakErazor (v1.76, Lighthouse Data) was used to filter contaminant peaks and when possible to perform internal calibration. Monoisotopic peptide masses were used for protein identification on Mascot (http://www.matrixscience.com/). Searches were done in MSDB database (3,239,079 sequences, 2006.08.31). Trypsin was set as the digestion enzyme and one missed cleavage site was allowed. A peptide tolerance of 50-100 ppm was considered and Cys carbamidomethylation and Met oxidation were set as fixed and variable amino acid modifications, respectively. A taxonomic restriction to mammalian protein sequences was included (339,504 sequences searched). The criteria used to accept the identification was significant homology scores achieved in Mascot (p < 0.05) and a minimum of five peptides matched allowing a minimum sequence coverage of 7%. Correspondence between theoretical mass and protein localization in the gel was also scrutinized. When MS spectra



Fig. 1 – (A) 2DE reference map of BHK cells metabolically labelled proteome, detected spots are outlined. (B) The 139 protein spots considered as differentially expressed are outlined (ANOVA test, p < 0.05; n = 4/5 gels/group, total n = 26, see Table 1SD of Supplemental data), gel localization and correspondent gene name of the 125 proteins identified by mass spectrometry are also indicated (see Table 2SD of Supplemental data). NI—not identified.

allowed the identification of two or more orthologous proteins with nearly the same probability and similar MW only the Rodentia protein with the best score is presented (taxonomic restriction to mammalian protein sequences was included in PMF analysis).

Most of the spots were analysed by combined MS+MS/MS. After overnight digestion with trypsin, supernatants were collected and extraction of tryptic peptides from the gel pieces was performed as described [15]. 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). Three S/N best precursors of each spectrum were selected for MS/MS analysis. The interpretation of the MS+MS/MS data was carried out by using the GPS Explorer software (Version 3.5, Applied Biosystems) and a local copy of the MASCOT search engine (Version 2.0). Monoisotopic peptide mass values were considered, a MS mass tolerance was set at 50 ppm and a MS/MS fragment tolerance set at 0.25 Da. Identification restrictions were the same as applied for PMF analysis. A taxonomic restriction to rodentia protein sequences was included. For MS all peaks with S/N greater than 5 and for MS/MS all peaks with S/N greater than 3 were searched against the Swissprot or NCBInr database (Swissprot 2008.06.09: total of 385,721 sequences, rodentia 24197; NCBInr 2008.06.20: total of 6,572,387 sequences, rodentia 221669). The criteria used to accept the identification was significant homology scores achieved in Mascot (p < 0.05).

3. Data analysis

Gene Ontology (www.geneonthology.org) was used to search for the biological processes, molecular functions and cellular components of the identified proteins [16]. PIKE (Protein Information and Knowledge Extractor) software was also used to collect all the available information about each protein (http://proteo.cnb.uam.es:8080/pike/).

Three clustering techniques [17–19] were applied to the spot volume data. An agglomerative unsupervised hierarchical clustering (Euclidean distance) was executed using geWorkbench version 1.5.1 (http://www.geworkbench.org), a free open source genomic analysis platform developed at Columbia University with funding from the NIH Roadmap Initiative





(1U54CA121852-01A1) and the National Cancer Institute. The Principal Component Analysis (PCA) and the Kohonen Self-Organizing maps (SOM) were employed using the software GenEx, trial version 4.3.5.514 (MultiD Analyses AB, Göteborg). For each of the 139 differentially expressed protein spots, when absent in a given phenotype, missing data were filtered and substituted by the remaining phenotypes arithmetic mean value for that spot. For scaling proposals all data were then normalised with the z-score algorithm.

In the correlation analysis of the different phenotypes studied, the Spearman rank correlation test was employed using the SPSS software (v.13.0 SPSS for Windows).

3.1. Western blot

Protein extracts from cell lines were quantified (Bradford; Bio-Rad protein assay) and 15 μ g of each cell sample (triplicates) were separated on 4–12% (w/v) polyacrylamide gels, transferred to nitrocellulose membranes (Schleicher & Schuell) and analysed by western blot. Membranes were probed with goat anti-Psme2 (Abcam), mouse anti-Cops5 (Abcam) or rabbit anti-RACK1 (Sigma-Aldrich) diluted 1:1000, 1:10,000 and 1:1000, respectively, in PBS plus 5% fat free milk for 2 h at RT and developed using enhanced chemiluminescence-ECL (GE Healthcare).



Fig. 3 – Unsupervised hierarchical clustering (Euclidean distances and average linkage) of the expression profiles of the 139 protein spots differentially expressed in the six phenotypes studied. Each horizontal row in the heat map represents the expression level for one protein in the different phenotypes (columns). The relative abundance is displayed by colour intensity (light red—more abundant; light green—less abundant).

Table 1 – Spearman's correlation analysis of the studied phenotypes.									
Correlations									
			F508del_37 °C	F508del_24h_26 °C	F508del_48h_26 °C	Wt_37 °C	Wt_24h_26 °C	Wt_48h_26 °C	
Spearman's rho	F508del_ 37 °C	Correlation coefficient	1.000	.566**	.715 **	.785 **	.520**	.519**	
		Sig. (2-tailed)	-	.000	.000	.000	.000	.000	
		Ν	133	133	130	128	129	130	
	F508del_ 24h_26 °C	Correlation coefficient	.566**	1.000	.855 **	.608 **	.849**	.831**	
		Sig. (2-tailed)	.000	-	.000	.000	.000	.000	
		Ν	133	139	135	133	135	135	
	F508del_ 48h_26 °C	Correlation coefficient	.715 **	.855 **	1.000	.698 **	.743**	.789**	
		Sig. (2-tailed)	.000	.000	-	.000	.000	.000	
		Ν	130	135	135	129	131	131	
	Wt_37 °C	Correlation coefficient	.785 **	.608 **	.698 **	1.000	.565 **	.564**	
		Sig. (2-tailed)	.000	.000	.000	-	.000	.000	
		Ν	128	133	129	133	129	130	
	Wt_24h_26 °C	Correlation coefficient	.520**	.849 **	.743 **	.565**	1.000	.941 **	
		Sig. (2-tailed)	.000	.000	.000	.000	-	.000	
		Ν	129	135	131	129	135	132	
	Wt_48h_26 °C	Correlation coefficient	.519**	.831**	.789 **	.564**	.941 **	1.000	
		Sig. (2-tailed)	.000	.000	.000	.000	.000	-	
		Ν	130	135	131	130	132	135	
** Correlation is significant at the 0.01 level (2-tailed).									

For western blots normalization, all membranes were washed with the stripping buffer [1.5% (w/v) Glycine, 0.1% SDS (w/v); 1% Tween 20 (v/v); pH 2.2] 5 times for 10 min, washed

with PBS and one last time with PBS-T. After, the membranes were probed as above with the mouse anti- tubulin (1:10,000) (Sigma-Aldrich) and developed using ECL. The abundance of

Fig. 4 – Graphical representation of the log normalized volume of proteins in BHK-wt (A) and BHK-F508del (B). BHK-wt and BHK-F508del at 37 °C expression levels of those proteins were considered as reference to determine the up or down-regulation of the correspondent protein in the other phenotypes, respectively in panels A and B. Proteins up and down-regulated in wt and F508del cells were also classified according to their molecular function.



Table 2 – BHK-wt and BHK-F508del cells response to low temperature treatment.						
Protein response	Proteins (gene name)					
wt-26 °C = F-26 °C	Psmc2, Cope, Srm, Mcm7, Gart, Umps [*] , Ogdh, Snd1, Pygb, spot 3443, Dpysl3 [*] , Eef2 (3422), spot 3421, Gfm1, Aco2 ^{**} , Qars [*] , Plod3, spot 3404 [*] , Vil2, Csde1, Snx6, Enoa (3394) [*] , spot 3359, spot 3357, Cct4 [*] , Adss, Eef1g, G6pdx [*] , Ublcp1 [*] , Ark7A2 [*] , Got1, Eif3a [*] , Anxa1 [*] , Psmd7 [*] , Pgam1 [*] , Prps2, Fabp5, Psmb7, Bdh1, Cdk4 [*] , Cops5 [*] , Prdx1, Ppia [*] , Pfn1, spot 3100 [*] , Prdx2, Psmb4, Npm1 (3083), Npm1 (3082), Npm1 (3081), Prdx6, Rexo2, Hspa9, Actb, Dlat ^{**} , Hnrnpk, G3bp1, HSPA8 (2992), HSPA8 (2988) [*] , Atp6v1a [*] , Atp5b, HSPA8 (2975) [*] , Ndufs1, Lonp1 [*] , spot 2937, Stmn1, Eif1ax ^{**} , Sh3bgrl [*] , Cbx1 [*] , Lgals1, Ube2n, Fth1, Nme1 [*] , Ubc [*] , Tmed3, Cmpk1 [*] , Tpt1 [*] , Enoa (2836), Pdia3 (2830), spot 2821, Pcna, Calu, Hspa5, Hsp90ab1, Vcp, Vcl, spot (2744), Cct1 ^{**} , Cct2					
wt-26 °C F-26 °C	Impdh2, Pdia3 (3425)*, Copd*, Ahcy1 (3299)**, Ahcy (3276)*, Actr2**, Vdac2, Gnb2l1**, Hist1h4a*, Psph**, spot 3020**, Txnd4**, Hnrpf**, Lamb1-1*, spot 2884*, Psme2*, Des*, Nap111, Rpsa*, Ccdc105*					
wt-26 °C # F-26 °C	Cnn3 (3538), Gdi2*, Pdlim1, Eef2 (3423), Pafah1b1, Adk*, Pnp, Tpi1, Psma6, Gstm5, S100a10, spot 3108*, Psmd2*, Ywhae*, spot 2881*, Snrpf, Cnn3 (2831), Fkbp9, Gdi1, Nasp, Plec1*, Ganab (2762), Ganab (2761)					

=, similar response to low temperature (up or down-regulated); _ , different response to low temperate; #, different magnitude of response (in one phenotype differences \geq 1.5 fold and in the other <1.5 fold); *, response observed in at least one of the incubation times (24 h or 48 h); **, spot not detected in one or more conditions.



the selected proteins and -Stubulin in those BHK cell lines was calculated from densitometry of immunoblots (n=3 replicates) using the Progenesis PG200v2006.

4. Results

4.1. 2DE proteome profiling

To identify proteins whose expression is modulated by low temperature that could be involved in the trafficking restoration process of F508del-CFTR, the total protein extracts from BHK cells stably expressing wt or F508del-CFTR grown at 37 °C or at 26 °C for 24 h or 48 h followed by 3 h of [³⁵S]-metabolic labelling were analysed by 2DE (for details see Materials and methods). A total of 26 2D-gel autoradiographic images were produced and the respective 2DE maps were computer-analysed. A total of 139 protein spots with significant differential expression (normalized volume differences above 1.5 fold and p ANOVA < 0.05) were detected when comparing the six conditions investigated: BHKwt 37 °C, BHK-wt 26 °C/24 h, BHK-wt 26 °C/48 h, BHK-F508del 37 °C, BHK-F508del 26 °C/24 h and BHK-F508del 26 °C/48 h (Fig. 1 and Table 1SD of Supplemental data). From those 139 protein spots we were able to identify 34 spots by PMF and 91 spots by MS+MS/MS combined analysis (Table 2SD, Supplemental data).

The correspondence between protein localization in the gel and their theoretical MW was consistent for the majority of the proteins identified. Some proteins, namely, nucleophosmin, calponin-3, neutral -glucosidase AB, plectin-1, elongation factor 2, Pdia3, adenosylhomocysteinase and Hsc70/Hspa8 were identified in different spots across the 2D gel suggesting for these proteins the presence of post-translational modifications or protein isoforms. In total, 125 protein spots, corresponding to 114 unique proteins plus 11 isoforms were identified.

Cellular localization, functional annotation and literature searches revealed that about 18% of the identified differential proteins are in the CFTR trafficking pathway compartments such as ER/ERGIC (ER Golgi intermediate compartment), Golgi, microssomes/endosomes and membrane/cell surface.

About 21% have protein folding and transport as molecular and biological functions and 12% are involved in protein degradation, namely in the proteasome system, as illustrated in Fig. 2 (Table 3SD, Supplemental data). Many of these proteins are known CFTR-interactors [20–22] such as ubiquitin, 26S proteasome subunits, Hsc70/Hspa8, GRP78/Hspa5, GRP75/mortalin/Hspa9, calumenin, Vcp, ezrin, etc, described to be involved in folding, trafficking, degradation and function of CFTR and also implicated in the cell quality control of some of these events (Tables 3SD, Supplemental data).

4.2. Clustering analysis

The protein expression patterns were analysed through unsupervised hierarchical clustering based on the hypothesis that proteins involved in the same physiological pathway/ mechanism, in response to the experimental conditions proposed, could have a similar expression profile. Fig. 3 shows the dendrogram resultant from agglomerative hierarchical clustering and the heat map of the 125 identified differentially expressed spots. The results obtained in the dendrogram (Fig. 3) were confirmed by Spearman's correlation analysis. Phenotypes at 37 °C were clustered apart from phenotypes at 26 °C, originating two different branches of the dendogram. The phenotypes constituting those clusters presented higher correlation coefficient among them comparing with the correlation coefficients obtained for phenotypes at 37 °C versus 26 °C (Table 1).

Among cells at low temperature, wt-CFTR expressing cells were more related to each other (higher coefficients) and the same happens with the F508del-CFTR expressing cells. Among the six phenotypes analysed, eight major protein clusters were then defined according to the heat map and sustained by PCA and the partitioning SOM analysis (data not shown). Through this approach, we searched for the possible involvement of some of those clusters or proteins in cells' differential response to low temperature treatment, which ultimately leads to F508del-CFTR trafficking rescue (see below).

4.3. Low temperature-induced stress response

The results (Fig. 4) demonstrated that incubation at low temperature induces great alterations in the protein expression profiles of the cells that is compatible with a cold-stress response. A significant decrease in proteins expression associated mainly with cell metabolism (23%/26%), transcription/translation (21%/23%) and degradation (12%/15%) were observed respectively in both BHK-wt and BHK-F508del cells in response to low temperature (Fig. 4). However, several other proteins presented enhanced expression under this treatment (e.g. see Fig. 3 clusters 1, 2, 3 and Fig. 4). Proteins up-regulated in both cells are mainly proteins involved in the regulation of transcription/translation (34%/38%), protein folding (21%/21%) and maturation and trafficking (13%/13%). The most relevant examples of these group of proteins are the cold-shock domain-containing protein E1 (Csde1) and the stress protein family, namely GRP75/mortalin/Hspa9, GRP78/BiP/Hspa5, Hsp90ab1/Hsp84 and three isoforms of heat shock proteins Hsc70/HspA8 (Fig. 4). Although with some differences between cells, i.e., 8% in BHK-wt and 13% in BHK-F508del, some proteins associated with ERAD degradation (e.g. Vcp) are also up-regulated in response to low temperature.

In which extension the response to cold-stress is different between BHK-F508del and BHK-wt cells that could predict CF cell recovery capacity from a stress situation were also investigated. Table 2 summarizes the results of this comparison indicating proteins with similar responses (89 proteins spots), different magnitude response (23 proteins spots) and antagonised responses (20 proteins) between cells at low temperature. This latter group is mainly associated with maturation and trafficking, transcription/translation, protein folding and cytoskeleton.

4.4. Protein expression modulation in BHK-F508del cells before and after low temperature treatment

At normal physiological growth conditions (37 °C), any protein that is down- or up-regulated in BHK-F508del cells in comparison to BHK-wt, is potentially associated with the quality control system responsible for ER trapping and degradation of F508del-CFTR. If, at induced trafficking rescue temperature, the expression of this protein is either rescued to



levels of BHK-wt cells, or in contrast, is even more exacerbated than in BHK-F508del, we can assume that this protein has a high probability to be involved in the trafficking and functional restoration process of F508del-CFTR achieved by this treatment. The heat map obtained (Fig. 3) illustrates a set of proteins included in this category that are discriminated in Table 3 (see also Table 4SD, Supplemental data). 18 of these proteins (Table 3A) were up-regulated in BHK-F508del cells but decreased their expression in response to low temperature. About 40% of these are associated with cell metabolism (e.g. Pygb and Dlat), 22% with cytoskeleton and transport (e.g. Vcl and Copd) and 16% with proteasome/protein degradation (e.g., 26S proteasome subunits).

In contrast, 10 proteins (Table 3B) which were downregulated in BHK-F508del increased their expression in direction to normal levels in response to low temperature. Most of them (60%) are associated with protein transport and folding (e.g. Pdia3 and Fkbp9), 20% with proteasome/protein degradation (e.g.19S non-ATPase subunit-Psmd2 and Vcp) and 10% with cytoskeleton (e.g. Actb).

Table 3C shows proteins that under low temperature enhance their expression difference in BHK-F508del in comparison with BHK-wt cells (e.g. Hnrnpk, Eef1g, Cope). 15 proteins were found belonging to this category, 23% are involved in protein folding/transport, 17% in metabolism, 9% in proteasome/protein degradation and 4% are structural constituents of cytoskeleton.

Given that any protein in BHK-F508del cells that modulates its expression in response to low temperature could be also involved in F508del-CFTR rescue process, we listed in Table 3D the group of proteins with that expression profile. About 68% of the 125 identified proteins were catalogued in this category, namely proteins associated with folding/transport/cytoskeleton (31%) (e.g. Calu and GRP78/BiP/Hspa5), metabolism (14%) and proteasome/protein degradation (8%).

The potential biological impact in the trafficking and functional restoration process of F508del-CFTR of the most relevant proteins herein identified is discussed below.

4.5. Biochemical validation by western blot analysis

The biochemical validation of 2DE-based proteomic results was performed by western blot analysis for three identified proteins of potential relevance for CFTR processing: Psme2 (Mr 27 kDa), the proteasome activator 28 subunit beta (PA28) [23]; Cops5 (Mr 37.7 kDa), a component of the COP9 signalosome [24], and RACK1 (Mr 35.5 kDa), a receptor activated C kinase [20] (alternative name: guanine nucleotide-binding protein subunit beta-2-like 1-Gnb2l1-). Alpha-tubulin (Mr 50 kDa) was examined in parallel as an internal housekeeping control. The expression levels were quantified in three independent experiments using densitometric analysis of immunoblots as shown in Fig. 5A, B and C.

The western blot analyses confirmed that Psme2 (Fig. 5A) is up-regulated in BHK-F508del cells, when compared with BHKwt, but its expression at 26 °C is lower than at 37 °C and decreased with the time of incubation; Cops5 and RACK1 (Fig. 5B and C, respectively) are both down-regulated in BHK-F508del cells at 37 °C when compared with BHK-wt at 37 °C. However RACK1 expression levels in BHK-F508del at 26 $^\circ\text{C}$ tend to return to BHK-wt levels, while Cops5 is even more down-regulated in BHK-F508del at 26 °C. Taking in consideration that western blot evaluates the steady state levels of proteins while the 2DE used in this study measures protein levels resultant from 'de novo' synthesis during the 3 h of metabolic labelling in the growth conditions studied, the obtained western blot results although not showing exactly the same expression differences as expected, were quite consistent and thus confirmed the 2DE data (Fig. 5D, E and F).

5. Discussion

To gain insight into the proteins potentially involved in the low temperature-induced F508del-CFTR rescue process, we explored in BHK-wt and BHK-F508del cells the global proteome modulation at 26 °C, with particular interest on the proteostasis environment [25] that may adequately support the processing of CFTR through the folding and trafficking pathways [1,26].

5.1. Low temperature induces cold-shock and unfolded protein response

Cell incubation at sub-optimal temperatures (25 °C–35 °C) is described as an inducer of a coordinated response involving modulation of cell cycle, metabolism, transcription, translation and cell cytoskeleton [27–30]. In accordance with this cold-shock response, we observed that at 26 °C the expression of several proteins, mainly cell-metabolism enzymes, proteins involved in transcription and translation (mostly associated with cell proliferation and DNA replication) and degradation (e.g. 20S proteasome subunits) are clearly down-regulated in both BHK cell lines (Fig. 4). In contrast, up-regulated proteins are mainly associated with transcription and translation (e.g. ribonucleoproteins), folding, maturation and trafficking (e.g. heat shock chaperones).

The cellular physiological effects of cold exposure are similar to those observed in heat-stress, which is characterised by the

Fig. 5 – Western blot validation (A, B, C) of 2DE analysis (D, E, F) of Psme2 (A, D), Cops5 (B, E) and RACK1 (C, F) protein expression in BHK-wt 37 °C, BHK-wt 26 °C/24 h, BHK-wt 26 °C/48 h, BHK-F508del 37 °C, BHK-F508del 26 °C/24 h and BHK-26 °C/48 h cells. The western blot graphics show the relative normalized abundance of Psme2 (A), Cops5 (B) and RACK1 (C) in those BHK cell lines calculated from densitometry of immunoblots using -tubulin as internal standard (n = 3 independent replicates/each Ab reaction). Close-up views of representative 2DE areas of Psme (D), Cops5 (E) and RACK1 (F) spots are shown. The 2DE graphics show the normalized % volume, i.e., the volume of each spot over the volume of all spots in the gel (ANOVA test, p < 0.05; n = 4/5 gels/group, total n = 26 gels). Progenesis PG200v2006 software (Nonlinear) was used for both western blot and 2DE densitometry analyses.

activation of molecular chaperones to assist in protein synthesis, unfolding/refolding, protein transport and many other cellular events [28]. In cold-stress response, however, cells over-express a small group of proteins termed CSPs [31–33], such as Csde1, a ribonucleoprotein involved in the regulation of transcription [34,35]. We confirmed here that, at 26 °C the Csde1 expression presented the typical modulation of the most known CSPs [29] since it is up-regulated in a positive correlation with cells incubation time from 24 h to 48 h (Table 1SD).

As the CSPs, Csde1 may also bind specifically to a limited number of RNA species, including several stress-inducible molecules [33]. Many of these Csde1 potential targets (e.g., Hsp90ab1/Hsp84, Hsc70/Hspa8, GRP78/BiP/Hspa5, GRP75/morta-lin/Hspa9, Vcp, Fkbp9) identified here were up-regulated under low temperature (Fig. 4 and Table 1SD). These proteins have also been associated with unfolded protein response (UPR) [36,37].

The UPR is a complex molecular cascade activated in response to cellular stressors, such as low temperature that is mainly characterised by up-regulation of ER chaperones to improve cell survival by facilitating proteins correct folding or assembly and preventing protein aggregation in ER lumen [36,37].

Other attempts using correctors, chemical chaperones and/ or mutagenic repair strategy (by inactivation of four RXR motifs of F508del-CFTR) (review in [38]) described to rescue F508del-CFTR from ER associated degradation (ERAD) to cell surface, cause UPR activation [39–41] [Gomes-Alves P et al, unpublished data]. These observations suggest an important role of UPR in the rescue process of F508del-CFTR.

However, over-expression of F508del-CFTR is also able to induce up-regulation of GRP78/BiP/Hspa5, a hallmarker of the UPR [42,43]. The 3 h of metabolic labelling used in this study was not sufficient to confirm this observation. Nevertheless, the UPR-induced by both low temperature treatment (this work) or mutagenic repair strategy [Gomes-Alves P et al, unpublished data] seems to involve a larger number of proteins in addition to GRP78/BiP/Hspa5. This UPR seems to be a critical mechanism able to generate a favourable cellular environment to promote F508del-CFTR rescue.

Singh et al. [21] demonstrated recently that the chemical rescue of F508del-CFTR mimics genetic repair in CF bronchial epithelial cells. Our temperature rescue approach appears to resemble some features of those repair strategies, namely the up-regulation of the ER stressors, GRP78/BiP/Hspa5 and GRP75/ mortalin/Hspa9. Regulation of other proteins, specifically Hsc70/Hspa8 in BHK cells at low temperature differs from what was observed in Singh's work probably due to the differences in the rescue strategies, techniques and cell lines used. Different key regulator proteins, like CFTR interacting chaperones, or different expression modulations of these proteins were also expected to be found considering the recent results of Rennolds et al. [8] suggesting that low temperature induces the appearance of a heterogeneous population of mature and immature CFTR molecules at the plasma membrane through different pathways.

5.2. Cold-shock response repairs some proteostasis imbalance in BHK-F508del cells

Here we confirmed our previous findings (Gomes-Alves P et al., unpublished data) showing that at normal physiological growth conditions, numerous proteins are differentially synthesised in BHK cells stably expressing F508del-CFTR (Table 3 and Table ISD). This suggests that different cellular mechanisms and/or pathways are being modulated in response to F508del-CFTR folding and trafficking defect. As Wang et al. revealed through a proteomic study, F508del-CFTR trapped in the ER sequesters more chaperones and co-chaperones than wt-CFTR [20].

Most of the identified proteins in this study are indeed located in the CFTR trafficking pathway compartments such as ER/ ERGIC, Golgi, microssomes/endosomes, membrane/cell surface. Several are described CFTR-interactors [20] involved in protein folding/transport (e.g. Hsp90ab1/Hsp84, Hsc70/Hspa8, GRP78/BiP/ Hspa5, GRP75/mortalin/Hspa9, TCP1-alpha/delta), or in protein degradation (e.g. proteasome subunits, Vcp) (see Table 3SD).

Here we also demonstrate that the low temperature stimulus introduces readjustments in the proteostasis imbalance associated with F508del-CFTR expression which probably endows the cell with the ability to partially rescue the mutant CFTR. An interesting observation in our study was that about 39% of the proteins differentially expressed at low temperature (F508del-CFTR expressing cells) were primarily found differentially expressed in BHK-F508del at physiological temperature (37 °C) in comparison with BHK-wt at the same conditions. Most of them (65%) reverts its expression levels under low temperature effect. Although the time and magnitude of expression response are different among these proteins, their reversion orchestration seems to be towards wt levels (Fig. 3 and Table 3), which is probably helping in the (partial) rescue of F508del-CFTR.

Some relevant proteins are described as CFTR-interactors and are associated with the proteasome and degradation (e.g. Psme2 and Cops5) or with protein folding and transport (e.g. RACK1) (Fig. 5D, E and F).

Psme2 (or PA28) is a constituent of the alternate proteasome cap (PA28) known as an ATP-independent activator implicated in the degradation of small peptides [23]. Shibatini et al. identified six mammalian cytosolic proteasome pools (20S, singly and doubly PA28-capped, singly 19S-capped, hybrid and doubly 19S-capped proteasomes/26S), showing that proteasomes are dynamic populations that undergo rapid exchange and/or redistribution of regulatory caps in response to changes in the cellular environment. Additionally four of those populations, the singly and doubly PA28-capped, hybrid and 26S proteasomes, interact with cytosolic CFTR degradation intermediates [23]. We observed that Psme2 is overexpressed in BHK-F508del at 37 °C in comparison to BHK-wt at 37 °C and BHK-F508del at 26 °C, which could be indicative of a significant role for this alternate regulatory cap in the degradation of BHK-F508del at 37 °C. On the other hand, Tanguy et al. [24] showed that Cops5 (also known by CSN5), a component of the COP9 signalosome (a complex that shares significant homologies with the 19S proteasome cap), associates with the core-glycosylated CFTR form. Moreover, overexpression of CSN5 (Cops5) caused a 25% decrease of F508del-CFTR while its down-regulation led to a decreased CFTR degradation rate, not enhancing however cell surface expression [24]. Some authors point COP9 signalosome as an alternative lid for the proteasome and named it COP9 proteasome [44,45]. We observed that Cops5 presents consistent down-regulation from BHK-F508del at 37 °C to 26 °C/24 h

to 26 °C/48 h, in comparison to BHK-wt. Cops5 shows a different modulation comparing to Psme2, however both proteins seem implicated in the CFTR degradative pathway and could probably give new clues on the regulation of CFTR turnover that seems to depend of a cooperative function of different sorting factors or proteasome subunits.

RACK1, detected in ER, COPI vesicles [46] and also in the membrane [47], is a CFTR interactor described to have a more robust interaction with wt-CFTR than with F508del-CFTR [20], moreover it may have an important role in the trafficking of CFTR as it was described by Auerbach and Liedtke that its down-regulation (by 77.5%) resulted in a significant decrease of apical expression of biotinylated CFTR (by 87.4%) [47]. They also propose a model for RACK1 interactions/functionality which includes PKC -RACK1-NHERF1-NHERF1-tubulin and suggest that it may play an important role in the stable expression of CFTR in the plasma membrane. We observed that, in contrast to what happened in BHK-wt cells, this protein was roughly not synthesised during 3 h of metabolic labelling in BHK-F508del at 37 °C. However, under low temperature treatment, the expression of this protein is recovered in the BHK-F508del cells.

6. Concluding remarks

By proteomics we showed that the cellular environment induced by low temperature that is favourable to (partial) rescue of F508del-CFTR processing and trafficking, is mainly characterised by cell-metabolism reduction and UPR activation. Numerous identified proteins have been described to be involved in protein folding and trafficking and many of them are located in the secretory pathway where mutated CFTR is retained.

At low temperature, several proteasome subunits presented different modulations reaffirming the dynamic and complex nature of this cellular structure. The down-regulation of proteasome subunits associated with CFTR degradation such as the regulatory PA28 caps and/or COP9 signalosome, an alternative lid for the proteasome, could be essential for (partial) F508del-CFTR rescue induced by low temperature.

Some proteostasis imbalance observed in BHK cells overexpressing F508del cells is recovered by low temperature treatment. The expression recovery of RACK1, a protein described as playing an important role in the stable expression of CFTR at the plasma membrane is a relevant example. Further studies will be, however, necessary to confirm and validate the involvement of those proteins in the rescue process of F508del-CFTR.

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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.jprot.2009.09.001.

REFERENCES

- [1] Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 1990;63:827–34.
- [2] Bush A, Alton EWFW, Davies JC, Griesenbach U. Cystic fibrosis in the 21st century. Switzerland: Karger; 2006.
- [3] Ward CL, Kopito RR. Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. J Biol Chem 1994;269:25710–8.
- [4] Denning GM, Anderson MP, Amara JF, Marshall J, Smith AE, Welsh MJ. Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature 1992;358:761–4.
- [5] Drumm ML, Wilkinson DJ, Smit LS, Worrell RT, Strong TV, Frizzell RA, Dawson DC, Collins FS. Chloride conductance expressed by delta F508 and other mutant CFTRs in *Xenopus* oocytes. Science 1991;254:1797–9.
- [6] Dalemans W, Barbry P, Champigny G, Jallat S, Dott K, Dreyer D, Crystal RG, Pavirani A, Lecocq JP, Lazdunski M. Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. Nature 1991;354:526–8.
- [7] Li C, Ramjeesingh M, Reyes E, Jensen T, Chang X, Rommens JM, Bear CE. The cystic fibrosis mutation (delta F508) does not influence the chloride channel activity of CFTR. Nat Genet 1993;3:311–6.
- [8] Rennolds J, Boyaka PN, Bellis SL, Cormet-Boyaka E. Low temperature induces the delivery of mature and immature CFTR to the plasma membrane. Biochem Biophys Res Commun 2008;366:1025–9.
- [9] Brown CR, Hong-Brown LQ, Biwersi J, Verkman AS, Welch WJ. Chemical chaperones correct the mutant phenotype of the delta F508 cystic fibrosis transmembrane conductance regulator protein. Cell Stress Chaperones 1996;1:117–25.
- [10] Haardt M, Benharouga M, Lechardeur D, Kartner N, Lukacs GL. C-terminal truncations destabilize the cystic fibrosis transmembrane conductance regulator without impairing its biogenesis. A novel class of mutation. J Biol Chem 1999;274:21873–7.
- [11] Roxo-Rosa M, Davezac N, Bensalem N, Majumder M, Heda GD, Simas A, Penque D, Amaral MD, Lukacs GL, Edelman A. Proteomics techniques for cystic fibrosis research. J Cyst Fibros 2004;3(Suppl 2):85–9.
- [12] Mendes F, Roxo Rosa M, Dragomir A, Farinha CM, Roomans GM, Amaral MD, Penque D. Unusually common cystic fibrosis mutation in Portugal encodes a misprocessed protein. Biochem Biophys Res Commun 2003;311:665–71.
- [13] Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 2004;25:1327–33.
- [14] Gobom J, Nordhoff E, Mirgorodskaya E, Ekman R, Roepstorff P. Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis

of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. J Mass Spectrom 1999;34:105–16.

- [15] Bensalem N, Masscheleyn S, Mozo J, Vallee B, Brouillard F, Trudel S, Ricquier D, Edelman A, Guerrera IC, Miroux B. High sensitivity identification of membrane proteins by MALDI TOF-MASS spectrometry using polystyrene beads. J Proteome Res 2007;6:1595–602.
- [16] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25:25–9.
- [17] Quackenbush J. Computational analysis of microarray data. Nat Rev Genet 2001;2:418–27.
- [18] D'Haeseleer P. How does gene expression clustering work? Nat Biotechnol 2005;23:1499–501.
- [19] Stahlberg A, Elbing K, Andrade-Garda JM, Sjogreen B, Forootan A, Kubista M. Multiway real-time PCR gene expression profiling in yeast Saccharomyces cerevisiae reveals altered transcriptional response of ADH-genes to glucose stimuli. BMC Genomics 2008;9:170.
- [20] Wang X, Venable J, LaPointe P, Hutt DM, Koulov AV, Coppinger J, Gurkan C, Kellner W, Matteson J, Plutner H, Riordan JR, Kelly JW, Yates III JR, Balch WE. Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. Cell 2006;127:803–15.
- [21] Singh OV, Pollard HB, Zeitlin PL. Chemical rescue of deltaF508-CFTR mimics genetic repair in cystic fibrosis bronchial epithelial cells. Mol Cell Proteomics 2008;7:1099–110.
- [22] Sun F, Hug MJ, Lewarchik CM, Yun CH, Bradbury NA, Frizzell RA. E3KARP mediates the association of ezrin and protein kinase A with the cystic fibrosis transmembrane conductance regulator in airway cells. J Biol Chem 2000;275:29539–46.
- [23] Shibatani T, Carlson EJ, Larabee F, McCormack AL, Früh K, Skach WR. Global organization and function of mammalian cytosolic proteasome pools: implications for PA28 and 19S regulatory complexes. Mol Biol Cell 2006;17:4962–71.
- [24] Tanguy G, Drévillon L, Arous N, Hasnain A, Hinzpeter A, Fritsch J, Goossens M, Fanen P. CSN5 binds to misfolded CFTR and promotes its degradation. Biochim Biophys Acta 2008;1783:1189–99.
- [25] Wang X, Koulov AV, Kellner WA, Riordan JR, Balch WE. Chemical and biological folding contribute to temperature-sensitive deltaF508 CFTR trafficking. Traffic; 2008.
- [26] Loo MA, Jensen TJ, Cui L, Hou Y, Chang XB, Riordan JR. Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. Embo J 1998;17:6879–87.
- [27] Thieringer HA, Jones PG, Inouye M. Cold shock and adaptation. Bioessays 1998;20:49–57.
- [28] Sonna LA, Fujita J, Gaffin SL, Lilly CM. Invited review: effects of heat and cold stress on mammalian gene expression. J Appl Physiol 2002;92:1725–42.
- [29] Al-Fageeh MB, Marchant RJ, Carden MJ, Smales CM. The cold-shock response in cultured mammalian cells: harnessing the response for the improvement of recombinant protein production. Biotechnol Bioeng 2006;93:829–35.

- [30] Al-Fageeh MB, Smales CM. Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. Biochem J 2006;397:247–59.
- [31] Graumann PL, Marahiel MA. A superfamily of proteins that contain the cold-shock domain. Trends Biochem Sci 1998;23:286–90.
- [32] Ermolenko DN, Makhatadze GI. Bacterial cold-shock proteins. Cell Mol Life Sci 2002;59:1902–13.
- [33] Fujita J. Cold shock response in mammalian cells. J Mol Microbiol Biotechnol 1999;1:243–55.
- [34] Hunt SL, Hsuan JJ, Totty N, Jackson RJ. unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. Genes Dev 1999;13:437–48.
- [35] Schepens B, Tinton SA, Bruynooghe Y, Parthoens E, Haegman M, Beyaert R, Cornelis S. A role for hnRNP C1/C2 and Unr in internal initiation of translation during mitosis. Embo J 2007;26:158–69.
- [36] Kelsen SG, Duan X, Ji R, Perez O, Liu C, Merali S. Cigarette smoke induces an unfolded protein response in the human lung: a proteomic approach. Am J Respir Cell Mol Biol 2008;38:541–50.
- [37] Citterio C, Vichi A, Pacheco-Rodriguez G, Aponte AM, Moss J, Vaughan M. Unfolded protein response and cell death after depletion of brefeldin A-inhibited guanine nucleotide-exchange protein GBF1. Proc Natl Acad Sci U S A 2008;105:2877–82.
- [38] Loo TW, Bartlett MC, Clarke DM. Rescue of folding defects in ABC transporters using pharmacological chaperones. J Bioenerg Biomembr 2005;37:501–7.
- [39] Obeng EA, Carlson LM, Gutman DM, Harrington Jr WJ, Lee KP, Boise LH. Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. Blood 2006;107:4907–16.
- [40] Hong M, Li M, Mao C, Lee AS. Endoplasmic reticulum stress triggers an acute proteasome-dependent degradation of ATF6. J Cell Biochem 2004;92:723–32.
- [41] Chaabane C, Corvazier E, Bredoux R, Dally S, Raies A, Villemain A, Dupuy E, Enouf J, Bobe R. Sarco/endoplasmic reticulum Ca2+ATPase type 3 isoforms (SERCA3b and SERCA3f): distinct roles in cell adhesion and ER stress. Biochem Biophys Res Commun 2006;345:1377–85.
- [42] Kerbiriou M, Le Drevo MA, Ferec C, Trouve P. Coupling cystic fibrosis to endoplasmic reticulum stress: differential role of Grp78 and ATF6. Biochim Biophys Acta 2007;1772:1236–49.
- [43] Bartoszewski R, Rab A, Jurkuvenaite A, Mazur M, Wakefield J, Collawn JF, Bebok Z. Activation of the unfolded protein response by DeltaF508 CFTR. Am J Respir Cell Mol Biol 2008;39:448–57.
- [44] Li L, Deng XW. The COP9 signalosome: an alternative lid for the 26S proteasome. Trends Cell Biol 2003;13:507–9.
- [45] Huang X, Hetfeld BK, Seifert U, Kahne T, Kloetzel PM, Naumann M, Bech-Otschir D, Dubiel W. Consequences of COP9 signalosome and 26S proteasome interaction. FEBS J 2005;272:3909–17.
- [46] Gilchrist A, Au CE, Hiding J, Bell AW, Fernandez-Rodriguez J, Lesimple S, Nagaya H, Roy L, Gosline SJ, Hallett M, Paiement J, Kearney RE, Nilsson T, Bergeron JJ. Quantitative proteomics analysis of the secretory pathway. Cell 2006;127:1265–81.
- [47] Auerbach M, Liedtke CM. Role of the scaffold protein RACK1 in apical expression of CFTR. Am J Physiol Cell Physiol 2007;293: C294–304.