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RESEARCH ARTICLE

Proteomic analysis of nasal cells from cystic fibrosis patients and non-cystic fibrosis control individuals: Search for novel biomarkers of cystic fibrosis lung disease

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Potential biological markers for cystic fibrosis (CF) lung disease were identified by comparative proteomics profiling of nasal cells from deletion of phenylalanine residue 508 (F508del)-homozygous CF patients and non-CF controls. From the non-CF 2-DE gels, 65 spots were identified by MS, and a reference 2-DE map was thus established. The majority of those correspond to ubiquitously expressed proteins. Consistent with the epithelial origin of this tissue, some of the identified proteins are epithelial markers (*e.g.* cytokeratins, palate lung and nasal epithelium clone protein (PLUNC), and squamous cell carcinoma antigen 1). Comparison of this protein profile with the one similarly obtained for CF nasal cells revealed a set of differentially expressed proteins. These included proteins related to chronic inflammation and some others involved in oxidative stress injury. Alterations were also observed in the levels of cytoskeleton proteins, being probably implicated with cytoskeleton organization changes described to occur in CF-airways. Lower levels were found for some mitochondrial proteins suggesting an altered mitochondrial metabolism in CF. Differential expression was also found for two more enzymes that have not been previously associated to CF. Further studies will clarify the involvement of such proteins in CF pathophysiology and whether they are targets for CF therapy.

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Abbreviations: CF, cystic fibrosis; **CFTR**, CF-transmembrane conductance regulator; **DTE**, 1,4-dithioerythritol; **F508del**, deletion of phenylalanine residue 508; **IL**, interleukin; **MGG**, May-Grünwald-Giemsa; **NFκB**, nuclear factor κB; **PDI A3**, protein disulfide isomerase A3; **PLUNC**, palate lung and nasal epithelium clone protein; **ROS**, reactive oxygen species; **SCCA1**, squamous cell carcinoma antigen 1; **%Vol**, volume of each spot over the volume of all spots in the gel

1 Introduction

Despite recent advances in our understanding of cystic fibrosis (CF) pathophysiology mechanisms, it remains the most common lethal, autosomal, recessive, genetic disease among Caucasians [1]. The gene, which when mutated is responsible for CF, encodes the CF-transmembrane conductance regulator (CFTR) protein, a cAMP-phosphorylation dependent-regulated chloride (Cl^-) channel. CFTR is expressed at the apical membrane of epithelial cells lining the airways, pancreatic ducts, intestine, biliary tree, sweat duct, and *vas deferens*

[1]. More than 1300 variants have been already reported to occur in the *CFTR* gene (http://www.genet.sickkids.on.ca/cftr). The most common severe CF-causing mutation, occurring in 70% of CF chromosomes worldwide, is the deletion of phenylalanine residue 508 (F508del) [1]. Due to misfolding, F508del-CFTR is directly sent to ER-associated degradation (ERAD) in the proteasome [2], thus failing to be processed and to reach the cell membrane [3].

Although this genetic disorder affects several organ systems, chronic obstructive lung disease is the major cause of morbidity and mortality among CF patients [4]. Indeed, the airways of these individuals are clinically characterized by mucostasis and mucus hypersecretion that cause small-airway obstruction with mucus plugging and air trapping. This is the propitious environment for successive bacterial infections, namely by *Pseudomonas aeruginosa*, resulting in chronic airway neutrophil-mediated inflammation, bronchiectasis, and ultimately, in patient death by lung failure [5]. Until now, therapy approaches for CF lung disease have been largely symptomatic, namely through antibiotic treatment against bacterial lung infection, and also with anti-inflammatory drugs [6]. The finding of novel therapeutic strategies that can efficiently overcome this pathology is even more complex as there is no straightforward correlation between CFTR genotype and CF lung disease severity [7]. Therefore, the identification of proteins whose expression is consistently affected in CF tissues may be of interest to help in the diagnosis, and perhaps prognosis, of this disorder, by acting as CF biomarkers.

Since the human nasal epithelium reflects the ionic proprieties of the characteristic CF dysfunction of the lower airways [6], we have used cells from the nasal epithelium to identify such CF markers. Moreover, human nasal cells can be easily obtained by a noninvasive procedure (*i.e.* nasal brushing), yielding numerous and well-preserved dissociated cells that are representative of the human superficial respiratory mucosa [8].

Thus, we obtained proteome profiles of nasal cells from non-CF individuals through a 2-DE-based proteomics approach, and compared them with those of F508del-homozygous CF patients. Proteins found to be consistently up- or downregulated in these native cells are potential biological markers of CF lung disease. Such proteins may also be proven to be prognosis factors and helpful in monitoring the evolution of the disease and/or following up the effect of therapies over time. Furthermore, assuming that these changes in protein levels correspond to those occurring in the lower airways, these disease-associated proteins may contribute in further studies to a better understanding of CF pathophysiology and become potential targets for novel therapeutic approaches.

2 Materials and methods

2.1 Individuals and samples collection

After informed consent, nasal cells were collected by the previously described brushing procedure [8, 9], from seven F508del-homozygous CF patients (two males, five females, mean age ± SD: 15.1 ± 6.6 years) in the absence of acute phase of respiratory inflammation and eight healthy, non-smoker and non-F508del-carrier volunteers (two males, six females, mean age ± SD: 28.8 ± 5.0 years) without nasal and respiratory infection. All CF patients were under established airway treatments, namely nebulized recombinant human dornase alpha (rhDNase) (Pulmozyme® inhalation solution, Roche, Basel, Switzerland), to reduce sputum viscoelasticity and Tobramycin (TOBI®, Chiron, Emeryville, CA, USA) to control respiratory infection and exacerbation.

Immediately after brushing, nasal cells were recovered from the brushes and washed twice in 1 mL of Opti-MEM medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) at room temperature (RT). Before pelleting the cells by centrifugation at 3000 rpm for 5 min in a Picofuge (Stratagene, La Jolla, CA, USA), an aliquot of 100 µL of the cellular suspension was saved for further May-Grünwald-Giemsa (MGG)-staining and cell examination by conventional light microscopy, as described elsewhere [9]. The pelleted cells, around 1×10^6 to 2×10^6 cells, were then quickly frozen and stored at −80°C until 2-DE analysis.

2.2 2-DE analysis

2-DE was performed as previously described [10]. Just prior to analysis, nasal cells were thawed and lysed in 100 µL of lysis buffer (7 M urea (Merck Diagnostic, Darmstadt, Germany), 2 M thiourea (Sigma-Aldrich Company, St. Louis, MO, USA), 2% w/v CHAPS (Sigma-Aldrich), 1% w/v 1,4-dithioerythritol (DTE) (Merck), 2% w/v ampholine 3.5–10.0 (Amersham Biosciences, Uppsala, Sweden), supplemented with a cocktail of protease inhibitors (1 mg/mL leupeptin, 2 mg/mL aprotinin, 121 mg/mL benzamidin, 50 mg/mL Pefabloc, 3.5 mg/mL E64). After addition of 250 µL of rehydration buffer (8 M urea, 2% w/v CHAPS, 10 mM DTE, 2% w/v ampholine 3.5–10.0 and a trace of bromophenol blue), the cell lysates were incubated for 1 h at 35°C. Samples were then clarified by centrifugation at $12\,000 \times g$ for 5 min and the supernatants containing the total extracted proteins were loaded onto 18 cm Immobiline DryStrips with a nonlinear wide-range pH gradient (pH 3–10). After active gel strip rehydration (30 V for 12 h at 20°C), IEF was run on an IPGphor IEF system (Amersham Bioscience) for a total of 100 kVh, during which the voltage was gradually increased up to 5000 V for a total of 50 h.

For SDS-PAGE, strips were incubated for 15 min at RT in equilibrating buffer (6 M urea, 50 mM Tris-HCl pH 6.8, 30% v/v glycerol, 2% v/v SDS, 2% w/v DTE) and then for another

15 min in blocking buffer (6 M urea, 50 mM Tris-HCl pH 6.8, 30% v/v glycerol, 2% v/v SDS, 2.5% w/v iodoacetamide (Sigma-Aldrich) and a trace of bromophenol blue) to reduce disulfide bonds of the proteins. Strips were finally applied onto 7–16% w/v gradient polyacrylamide gels and run overnight at 2.5 W/gel (Ettan DALT*twelve* System, Amersham Biosciences).

After electrophoresis protein visualization was carried out by silver staining, compatible to MS analysis or CBB-staining as previously described (Roxo Rosa, M., Amaral, M. D., Penque, D., *The Virtual Repository of Biological Methods and Reagents for CFTR Expression and Functional Studies* (http://central.igc.gulbenkian.pt/cftr/vr/biochemistry.html) Section C. 2004).

2.3 2-DE maps analysis

The digitalized images of the silver-stained 2-DE gels were obtained using ImageScanner (Amersham Biosciences) and these were analyzed using the ImageMaster™ 2-D Platinum software. In order to standardize the intensities of silver staining among spots present in several 2-DE maps, analysis was carried out by taking into account the standardized relative intensity volume of spots (or %Vol, *i.e.* the volume of each spot over the volume of all spots in the gel). The presence of mucus in our samples interfered with the staining of the 2-DE gels; therefore, their analysis was performed only in the area of the gel for which the spots were always detected, *i.e.* below ~60 kDa. The difference in expression levels between nasal cells from CF patients and healthy controls for a given protein was statistically assessed by using the two-sample *t*-test, for n observations, where n is the total number of individuals analyzed (CF + non-CF). Differences were considered statistically significant for $p < 0.05$.

2.4 Sample preparation for MS analysis

2.4.1 CBB-stained gels

Protein spots from the CBB-stained 2-DE gels were destained with 30% ACN in 0.1 M ammonium hydrogen carbonate (Merck) and washed with Millipore water. The gel pieces were lyophilized by a Speedvac evaporator (Savant, Farmingdale, USA) for 30–60 min, followed by digestion with 0.4 μg (2U) trypsin (Promega, Madison, WI, USA) in 4 μl of 3 mM Tris-HCl (pH 8.8), overnight at RT. The peptide mixture was extracted from the gel plug with 7 μL of a 33% ACN + 0.1% TCA (TFA) solution.

2.4.2 Silver-stained gels

Protein spots from the silver-stained 2-DE gels were destained for 20 min in the dark with 200 μL of a 1:1 solution of 30 mM potassium hexacyanoferrate(III) and 100 mM sodium thiosulphate. Before drying the gel pieces in a Speedvac evaporator, they were washed with Millipore water and shrunk in the presence of ACN for 30 min. Trypsin digestion was performed as described previously for CBB-stained gels. Digested peptides present in the supernatants were cleaned and concentrated using Porous R12 microcolumns (Applied Biosystems, Foster City, CA, USA).

2.5 Protein identification by PMF

2.5.1 CBB-stained gels

Peptide mixture of 0.5 μL was mixed on a MALDI target with 0.5 μL of saturated matrix solution consisting of CHCA (Merck, twice crystallized) resuspended in 1 mL of ACN. PMF was assessed by MALDI-TOF-MS analysis, which was performed on a Biflex™ III (Bruker Daltonik, Bremen, Germany) equipped with a SCOUT 384 XY table. An anchorchip with 400 μm anchors (Bruker Daltonik) was used to measure the peptide profiles. Data acquisition was performed on a SUN Ultra using XACQ software, version 5.1. For external calibration a standard peptide mixture was used (Sigma-Aldrich).

2.5.2 Silver-stained gels

Digested peptides were eluted with 0.8 μL of 10 mg/mL CHCA, prepared in 70% ACN v/v and 0.1% v/v TFA directly on top of the MALDI target. Sample peptides were assayed for PMF in a Voyager-DE™ STR MALDI-TOF mass spectrometer (Applied Biosystems). The mixture was allowed to air dry in order to promote cocrystallization. Spectra were acquired using the Voyager Instrument Control Panel Software, version 5.10. Data Explorer version 4.4 was used for spectra processing; m/z peak evaluation was performed by MoverZ (ProteoMetrics, New York, USA). External calibration was performed using peptide calibration Mix1 (LaserBio Labs, France).

In both cases PeakErazor (Lighthouse Data, Odense, Denmark) was used to filter contaminant m/z peaks and, when possible, to perform internal calibration. Monoisotopic peptide masses were used to search for homologies and protein identification with PMF software of MASCOT (http://www.matrixscience.com). Searches were done in MSDB (MS protein sequence DataBase) database (http://csc-fserve.hh.med.ic.ac.uk/msdb.html). A mass accuracy of 50–100 ppm was considered for external calibrations and Cys carbamidomethylation and Met oxidation as fixed and variable amino acid modifications, respectively. A taxonomic restriction to mammalian protein sequences was included. Criteria used to accept the identification were significant homology scores achieved in MASCOT, and a minimum of four peptides matched, allowing a protein sequence coverage above 10%.

2.6 Computational analysis of the biological association among proteins

The possible biological relationship among the proteins that were found to be differentially expressed between CF and non-CF nasal cells was assessed by using the PathwayAssist™ version 3.0 software.

3 Results

3.1 Validation of nasal cell samples

As previously described [9], the great majority (around 80–95%) of the nasal cells collected by brushing are well-preserved and dissociated epithelial cells, even when collected from CF patients (data not shown). This can be easily observed by MGG staining (Fig. 1A), revealing the presence of three major cell types according to previously described classification criteria [9], namely tall columnar (ciliated and nonciliated) cells, secretory goblet cells, and basal cells. Moreover, in agreement to the previously described evaluation of the cellular types recovered by nasal brushing [9], we did not found any significant difference in the cellular type distribution between CF and non-CF cell samples, except in the number of inflammatory cells which are more abundant in CF (~8%) than in non-CF samples (~1–3%) (data not shown).

3.2 Computer-assisted analysis of the 2-DE gels

Total soluble protein extracts of healthy non-CF ($n = 8$) and F508del-homozygous CF ($n = 7$) nasal cells were separated by 2-DE (Figure see Suppl. Material). After silver staining, the digitalized images of the gels were analyzed using a specific Proteomics software. We observed that on average the number of spots detected *per* gel on 2-DE gels from CF cells ($n = 541$) is not significantly different from the number detected on 2-DE gels from non-CF (control) nasal cells ($n = 591$). The different appearance of the 2-DE maps obtained for the two groups of individuals under comparison (Fig. 2A *vs.* B) is explained by the fact that the protein separation of CF nasal cell samples was affected by excess mucus (Fig. 1B). Moreover, this excess mucus makes the collection of cells by brushing more difficult in CF patients, causing a slight reduction in the number of cells obtained in these samples in comparison to those of non-CF individuals. To overcome these technical problems and to obtain reliable gel-to-gel matching analysis, the identity of some of the same matched spots ($n = 19$) was confirmed by MS in several 2-DE gels.

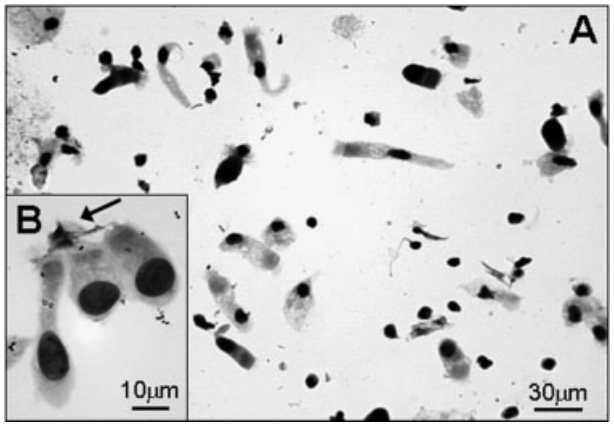

Figure 1. Nasal cells collected by brushing. (A) MGG-staining of nasal cells sample from a F508del-homozygous CF patient. As in samples collected from non-CF controls, the majority of the cells are epithelial. (B) A thick mucus layer (indicated by a black arrow) can readily be observed in a group of CF tall columnar cells.

After this preliminary procedure, computer-assisted analysis was carried out taking into account the %Vol. Through heuristic clustering analysis, *i.e.* an artificial intelligence-based analysis to automatically classify sets of gels into different classes according to their characteristic spots, the 2-DE gels ($n = 8 + 7$) were automatically (and correctly) separated into two distinct classes. It is important to note that the referred differences in appearance did not interfere with the heuristic clustering, as long as the preliminary gel-to-gel matching was done. After statistical evaluation of the variations of %Vol for spots between the two classes of maps, analysis has shown that there are at least 18 spots with significantly different expression ($p < 0.05$) levels between CF and non-CF nasal samples. Figure 3 gives an example of the computer-assisted analysis for two given proteins, a less expressed protein (Fig. 3A) and another expressed at higher levels (Fig. 3B) in nasal cell 2-DE gels of CF patients *versus* controls.

3.3 Nasal cells proteome profile

The 2-DE maps obtained for all samples were found to be reproducible and consistent, allowing the establishment of a reference 2-DE map of nasal cells from non-CF individuals (Fig. 2A). From this 2-DE proteome profile, 65 of the most representative spots were analyzed, following trypsin digestion, by PMF, using MALDI-TOF-MS spectrometers (Fig. 2A and Table 1). As mentioned above, 19 of these spots were identified in several 2-DE gels, further supporting the computer-assisted analysis of the gels.

The majority of protein spots identified ($n = 40$) were found to be ubiquitously expressed proteins (Table 1). These include proteins in functional classes, such as: (1) molecular chaperones (binding protein (BiP) (spot 13), calreticulin (spot 15), 75 kDa glucose regulated protein (GRP75) (spot 26), Hsc70 (spot 29), Hsp60 (spot 31), Hsp70 (spot 32), protein disulfide isomerases (spots 48, 49a, and 49b)); (2) cytoskeleton constituents (β-actin (spot 12), tubulins α1 (spot 56), α6 (spot 57), β (spot 58)); (3) several enzymes (α-enolase (spot 3), ATP synthase β chain (spot 10), superoxide dismutase-Mn (SOD-Mn) (spot 53), *etc.*); and also (4) proteins that regulate cellular processes

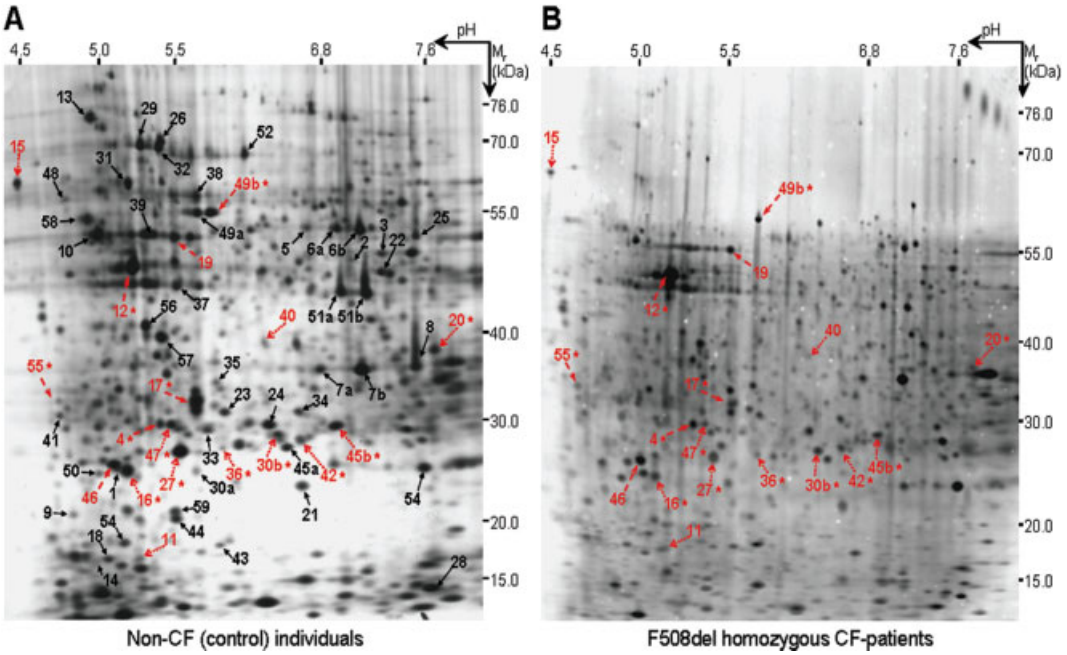

Figure 2. 2-DE reference maps of protein profiles of human nasal cells, from (A) non-CF controls and (B) F508del-homozygous CF patients. Proteins from $\sim 1 \times 10^6$ to 2×10^6 nasal cells recovered by brushing were separated as described in Section 2. After MS-compatible silver staining, gels were analyzed using ImageMaster 2-D Platinum software (see Section 2). About 600 spots were detected, of which 65 were identified by MS analysis. Spot numbers indicated in the 2-DE maps are the same as used for MS identification, as in Table 1. Differences in expression levels of proteins between CF and non-CF nasal cells, observed among the various 2-DE maps, were statistically assessed. Black arrows indicate spots with similar expression levels between the two groups ($p > 0.05$) or spots for which statistical analysis was not possible. Red arrows indicate spots exhibiting different expression levels in CF *vs.* non-CF nasal cells (more abundant; less abundant). Significant differences ($p < 0.05$) are indicated by an asterisk.

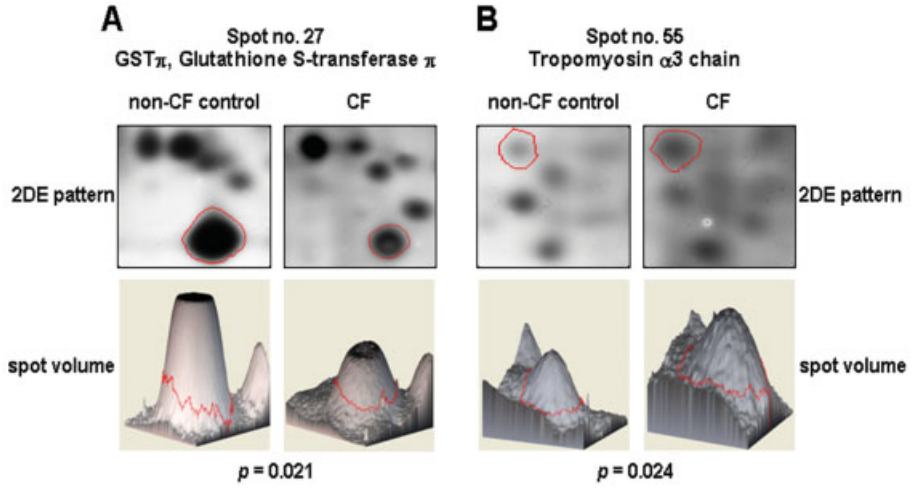

Figure 3. ImageMaster 2D Platinum output showing two representative differentially expressed spots ($p < 0.05$) between human nasal cells from CF patients and non-CF controls. (A) Spot no. 27, GSTπ, expressed in lower levels ($p = 0.021$) in CF ($n = 7$) than in non-CF controls ($n = 8$) nasal cells. (B) Spot no. 55, tropomyosin α3 chain, more abundantly expressed ($p = 0.024$) in CF ($n = 7$) than in non-CF control ($n = 8$) nasal cells. For each spot an enlarged region of the respective 2-DE reference maps is shown which contains the referred spot (highlighted in red) and the corresponding spot volume. Each spot was identified by MALDI-TOF MS analysis.

Table 1. Identification of 65 spots from 2-DE protein profiles of human nasal cells, by MALDI-TOF-MS analysis. Black points (•) indicate M_r of a fragment of the protein, and two black points (••) indicate M_r of the corresponding unprocessed precursor

Spot number	Protein	Gene	Accession number	Theoretical		Subcellular localization	Spot visualization	Sequence coverage (%)
				p*I*	M_r (Da)			
1	3′(2′),5′-bisphosphate nucleotidase 1	BPNT1	O95861	5.46	33 392	Cytoplasm	Silver	27
2	Acyl-CoA dehydrogenase	ACADM	P11310	7.02	43 643	Mitochondrial matrix	Silver	12
3	α-Enolase	ENO1	P06733	6.99	47 037	Cytoplasm	Silver	30
4	Alcohol dehydrogenase ($NADP^+$)	AKR1A1	P14550	6.35	36 442	Cytoplasm	Silver	15
5	Aldehyde dehydrogenase 12	ALDH12	Q9H2A2	6.76	53 401	Cytoplasm	Silver	15
6 (a, b)	Aldehyde dehydrogenase 1A1	ALDH1A1	P00352	6.29	54 730	Cytoplasm	Coomassie	36, 32
7 (a, b)	Annexin A1	ANXA1	P04083	6.64	38 583	Plasma membrane	Coomassie	25, 25
8	Annexin A2	ANXA2	P07355	7.56	38 472	Lamina beneath the plasma membrane	Silver	37
9	Annexin A5	ANXA5	P08758	4.94	35 806	Cytoplasm	Silver	36
10	ATP synthase B chain	ATP5B	P06576	5.00	51 769	Mitochondria	Coomassie	34
11	ATP synthase D chain	ATP5H	O75947	5.22	18 360	Mitochondria	Silver	45
12	β-Actin	b-Actin	Q96HG5	–	41 005•	Cytoskeleton	Coomassie	28
13	BiP protein, GRP78	HSPA5	Q9UK02	–	70 931•	ER	Coomassie	33
14	Calcyphosine	CAPS	Q13938	4.74	20 967	Cytoplasm	Silver	57
15	Calreticulin	CALR	P27797	4.29	46 466	ER lumen	Silver	13
16	Cathepsin B	CTSB	P07858	5.23	37 808••	Lysosomes	Silver	41
17	Cathepsin D	CTSD	P07339	5.60	37 852	Lysosomes	Silver	41
18	CENP-F kinetochore protein, centromere protein F	CENPF	P49454	5.03	367 594	Nuclear matrix	Silver	40
19	Desmin	DES	P17661	5.21	53 405	Cytoplasm	Silver	37
20	Dihydrodiol dehydrogenase 2	AKR1C2	P52895	7.13	36 735	Cytoplasm	Silver	36
21	DJ-1 protein, RNA binding protein regulatory subunit	PARK7	O14805	6.33	19 891	Nucleus and cytoplasm	Silver	53
22	Elongation factor Tu	TUFM	P49411	6.31	45 045	Mitochondria	Silver	21
23	Endoplasmatic reticulum protein, ERp29	ERP29	P30040	6.08	25 853	ER lumen	Silver	32
24	Enoyl-CoA hydratase	ECHS1	P30084	5.88	28 355	Mitochondrial matrix	Silver	44
25	Glutamate dehydrogenase 2	GLUD2	P49448	6.74	56 053	Mitochondrial matrix	Silver	20
26	GRP75, Stress-70 protein	HSPA9B	P38646	5.44	68 759	Mitochondria	Silver	17
27	GSTp	GSTP1	P09211	5.44	23 342	Cytoplasm	Coomassie	62
28	Hemoglobin b subunit variant (fragment)	HBB	Q9UK54	–	13 964•	Cytoplasm	Coomassie	58
29	Hsc70, Heat shock cognate 71 kDa protein	HSPA8	P11142	5.37	70 898	Cytoplasm, nucleus (HS response)	Coomassie	36
30 (a, b)	Hsp27, heat-shock protein b-1	HSP1	P04792	5.98	22 783	Cytoplasm, nucleus (HS response)	Silver	34, 32
31	Hsp60, heat shock protein 60 kDa	HSPD1	P10809	5.24	57 963	Mitochondrial matrix	Coomassie	18
32	Hsp70, heat shock 70 kDa protein 1	HSPA1A	P08107	5.48	70 052	Cytoplasm, nucleus (HS response)	Silver	22
33	Hypothetical protein FLJ20772	FLJ20772	Q6PKB9	–	42 066	Unknown	Silver	16
34	Hypothetical protein FLJ21628	Unknown	Q9H6Z6	9.24	44 950	Nucleus	Silver	18
35	Hypothetical protein FLJ23514	Unknown	Q9H5E5	9.08	45 484	Unknown	Silver	25
36	Hypothetical protein FLJ43998	Unknown	Q6ZU50	5.88	90 341	Plasma membrane	Silver	32
37	Keratin, type I cytoskeletal 19	KKRT19	P08727	5.05	44 106	Cytoskeleton	Coomassie	37
38	Keratin, type II cytoskeletal 5	KRT5	P13647	8.14	62 447	Cytoskeleton	Silver	24
39	Keratin, type II cytoskeletal 8	KRT8	P05787	5.52	53 543	Cytoskeleton	Coomassie	32
40	MAX protein	MAX	Q6V3B1	5.82	11 026	Nucleus	Silver	22
41	MYO1G protein	MYO1G	Q96BE2	–	33 978•	Cytoplasm	Silver	15
42	NADH-ubiquinone oxidoreductase 30 kDa subunit	NDUFS3	O75489	5.48	26 415	Mitochondrial inner membrane	Silver	40
43	Peroxiredoxin 1	PRDX1	Q06830	8.27	22 110	Cytoplasm, peroxisome	Coomassie	55
44	Peroxiredoxin 2	PRDX2	P32119	5.66	21 892	Cytoplasm, peroxisome	Silver	46
45 (a, b)	Peroxiredoxin 6	PRDX6	P30041	6.02	24 904	Cytoplasm, lysosomes	Silver	32, 46
46	PLUNC	PLUNC	Q9NP55	5.42	24 669	Secreted	Silver	35
47	Prohibitin	PHB	P35232	5.57	29 804	Mitochondria	Silver	37
48	PDI	P4HB	P07237	4.69	55 294	ER lumen	Silver	32
49 (a, b)	PDI A3	PDIA3	P30101	5.61	54 265	ER lumen	Silver	23, 17

Table 1. Continued

Spot number	Protein	Gene	Accession number	Theoretical		Subcellular localization	Spot visualization	Sequence coverage (%)
				p*I*	M_r (Da)			
50	Rho GDP-dissociation inhibitor 1	ARHGDIA	P52565	5.03	23 207	Cytoplasm	Silver	25
51 (a, b)	SCCA1	SERPINB3	P29508	6.35	45 565	Cytoplasm	Coomassie	41, 40
52	Serum albumin	ALB	P02768	5.67	66 472	Secreted	Coomassie	28
53	SOD-Mn	SOD2	P04179	6.86	22 204	Mitochondrial matrix	Coomassie	38
54	Sorcin	SRI	P30626	5.32	21 676	Cytoplasm	Silver	30
55	Tropomyosin a3 chain	TPM3	P06753	4.68	32 818	Cytoskeleton	Coomassie	27
56	Tubulin α1 chain	TUBA1	P05209	4.94	50 136	Cytoskeleton	Silver	24
57	Tubulin α6 chain	TUBA6	Q9BQE3	4.96	49 895	Cytoskeleton	Coomassie	22
58	Tubulin β chain	TUBB1	P07437	4.78	49 671	Cytoskeleton	Coomassie	27
59	UMP-CMP kinase	UCK	P30085	5.44	22 222	Nucleus (mainly), cytoplasm	Silver	60

(annexins A1 (spots 7a and 7b) and A2 (spot 8); DJ-1 protein or RNA binding protein regulatory subunit (spot 21), *etc.*).

During the nasal brushing procedure some local bleeding may occur. Therefore, the identification of proteins characteristic of blood cells, *e.g.* hemoglobin β subunit variant (fragment) (spot 28) from red blood cells, is not surprising. Nevertheless, the epithelial origin of most cells obtained by that procedure was confirmed through the identification of epithelium-specific proteins, namely keratins (type I cytoskeletal 19 (spot 37), type II cytoskeletal 5 (spot 38), and 8 (spot 39)) [11], palate lung and nasal epithelium clone protein (PLUNC) (spot 46) [12], and squamous cell carcinoma antigen 1 (SCCA1) (spots 51a and 51b) [13].

Within the same 2-DE gel, some different spots were identified by MS as being the same protein (*viz.*, aldehyde dehydrogenase 1A1–spots 6a and 6b; annexin A1–spots 7a and 7b; Hsp 27–spots 30a and 30b; peroxiredoxin 6–spots 45a and 45b; protein disulfide isomerase A3 (PDI A3)–spots 49a and 49b; and SCCA1–spots 51a and 51b). Further studies are required to confirm whether these are isoforms of the same protein resulting from PTMs (*viz.* phosphorylation, glycosylation, *etc.*).

3.4 Differential protein expression profile in CF nasal cells

Figure 2B shows a typical 2-DE map obtained for F508del-homozygous CF nasal cells. From the referred 18 differentially expressed spots, 13 were identified by MS (Tables 1 and 2) (indicated with red arrows and asterisks in Fig. 2A and B). There are five additional spots, also identified by MS, for which expression appears to be altered, although not significantly ($p > 0.05$) (Table 2).

We grouped these differentially expressed proteins according to their cellular function into six classes: (1) chronic inflammation (cathepsins B (spot 17) and D (spot 18), Hsp27 (spot 30b), and PLUNC (spot 46)); (2) oxidative stress (GSTπ (spot 27), and peroxiredoxin 6 (spot 45b)); (3) cytoskeleton organization (β-actin (spot 12), desmin (spot 19), tropomyosin α3 chain (spot 55)); (4) molecular chaperones (calreticulin (spot 15) and PDI A3 (spot 49b)); (5) metabolic functions (alcohol dehydrogenase ($NADP^+$) (spot 4), ATP synthase D chain (spot 11), dihydrodiol dehydrogenase 2 (spot 20), NADH-ubiquinone oxidoreductase 30 kDa subunit (spot 42), prohibitin (spot 47)); and (6) unknown (hypothetical protein FLJ43998 (spot 36), MAX protein (spot 40)).

In order to find a relationship among these differentially expressed proteins and CFTR, we built their regulatory (Fig. 4A) and biological function (Fig. 4B) pathways, using a specific software that correlates the scientific information of each protein. As expected, we can observe from these highly complex pathways that there are many cellular mechanisms connecting those proteins to CFTR, suggesting that, indeed, dysfunction of CFTR could have an influence on their expression patterns, either directly or through regulatory effects, *etc.* However, as some of the differentially expressed proteins between CF and non-CF nasal cells were not integrated in such pathways (*viz.*, alcohol dehydrogenase ($NADP^+$) (spot 4), ATP synthase D chain (spot 11), dihydrodiol dehydrogenase 2 (spot 20), hypothetical protein FLJ43998 (spot 36), MAX protein (spot 40), NADH-ubiquinone oxidoreductase 30 kDa subunit (spot 42), PLUNC (spot 46), and tropomyosin α3 chain (spot 55)), subsequent studies will be required in order to clarify the roles of each in CF pathophysiology. A more restricted regulatory pathway was built (Fig. 4C) to evaluate the involvement of proteases, (*e.g.*, cathepsins B and D, spots 16 and 17), and their inhibitors, (*e.g.*, SCCA1, spots 51a and 51b) during the CF chronic inflammation process.

Table 2. Proteins found to be differentially expressed in nasal cells from CF patients ($n = 7$) *versus* non-CF controls ($n = 8$), by using the two-sample *t*-test. For each spot the average of the %Vol ± M.S.D. (mean of SD) is indicated. "CF *vs.* non-CF" column indicates the ratio values between %Vol average of spots on CF 2-DE maps and %Vol average of the same spots on non-CF 2-DE maps

Spot no.	Protein	% Vol average CF ± M.S.D.	% Vol average nonCF ± M.S.D.	CF *vs.* non-CF	*p* value	Cellular function
4	Alcohol dehydrogenase ($NADP^+$)	0.303 ± 0.148	0.109 ± 0.037	2.774	0.023*	Belongs to the aldo/keto reductase family. Catalyzes the NADPH-dependent reduction of a variety of aldehydes to their corresponding alcohols.
11	ATP synthase D chain	0.264 ± 0.105	0.646 ± 0.222	0.408	0.058	This is one of the chains of the nonenzymatic component of the mitochondrial ATPase complex. It has no apparent bacterial homolog and its exact function is unknown.
12	β-Actin	1.297 ± 0.435	0.497 ± 0.126	2.612	0.049*	Component of cell cytoskeleton.
15	Calreticulin	0.112 ± 0.051	0.183 ± 0.102	0.614	0.068	Molecular Ca^{2+}-binding chaperone.
16	Cathepsin B	0.415 ± 0.072	0.655 ± 0.029	0.633	0.002*	Thiol-protease that has been implicated in tumor invasion and inflammation.
17	Cathepsin D	0.460 ± 0.089	0.147 ± 0.061	3.122	0.013*	Acid protease which is estrogen inducible, may have a role during the pathogenesis of breast tumors.
19	Desmin	0.369 ± 0.153	0.133 ± 0.057	2.764	0.053	Belongs to the intermediate filament family.
20	Dihydrodiol dehydrogenase 2	0.082 ± 0.045	0.287 ± 0.027	0.287	0.003*	Belongs to the aldo/keto reductase family. Catalyze the conversion of aldehydes and ketones to their corresponding alcohols by utilizing NADH and/or NADPH as cofactors.
27	GSTp	0.559 ± 0.100	1.544 ± 0.509	0.362	0.021*	GSTs are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione.
30b	Hsp27, Heat-shock protein b-1	0.210 ± 0.027	0.375 ± 0.094	0.560	0.033*	Involved in stress resistance and actin organization.
36	Hypothetical protein FLJ43998	0.083 ± 0.024	0.177 ± 0.018	0.468	0.003*	Belongs to the cation transport ATPases family (P-type ATPases).
40	MAX protein	0.098 ± 0.017	0.178 ± 0.051	0.551	0.087	Unknown/transcription regulator.
42	NADH-ubiquinone oxidoreductase 30 kDa subunit	0.113 ± 0.044	0.251 ± 0.080	0.451	0.040*	Transfer of electrons from NADH to the respiratory chain.
45b	Peroxiredoxin 6	0.124 ± 0.032	0.324 ± 0161	0.382	0.039*	Involved in redox regulation of the cell.
46	PLUNC	1.309 ± 0.580	0.250 ± 0.091	5.230	0.074	Airway inflammatory response after exposure to irritants. Specifically expressed in upper airways.
47	Prohibitin	0.094 ± 0.027	0.250 ± 0.114	0.376	0.015*	Inhibits DNA synthesis. It has a role in regulating proliferation. It is involved in the assembly of mitochondrial respiratory chain complexes.
49b	PDI A3	0.301 ± 0.078	0.192 ± 0.052	1.566	0.028*	Rearrangement of disulfide bonds in proteins to form their native structures.
55	Tropomyosin α3 chain	0.158 ± 0.006	0.062 ± 0.022	2.539	0.024*	Binds to actin filaments in muscle and nonmuscle cells. In nonmuscle cells it is implicated in stabilizing cytoskeleton actin filaments.

* statistically significant differences ($p < 0.05$)

4 Discussion

Through the comparison of protein profiles of nasal cells from F508del-homozygous CF patients and non-CF controls, we identified here a set of proteins that are differentially expressed. We now discuss the possible involvement of such proteins in CF lung disease.

4.1 Chronic inflammation

Recurrent and persistent bacterial infections of CF lung, namely by *P. aeruginosa*, lead to a neutrophil-mediated chronic inflammation [4], which results from an impaired balance between the activity of proteases and their respective inhibitors [14]. Interestingly, our results indicate an altered

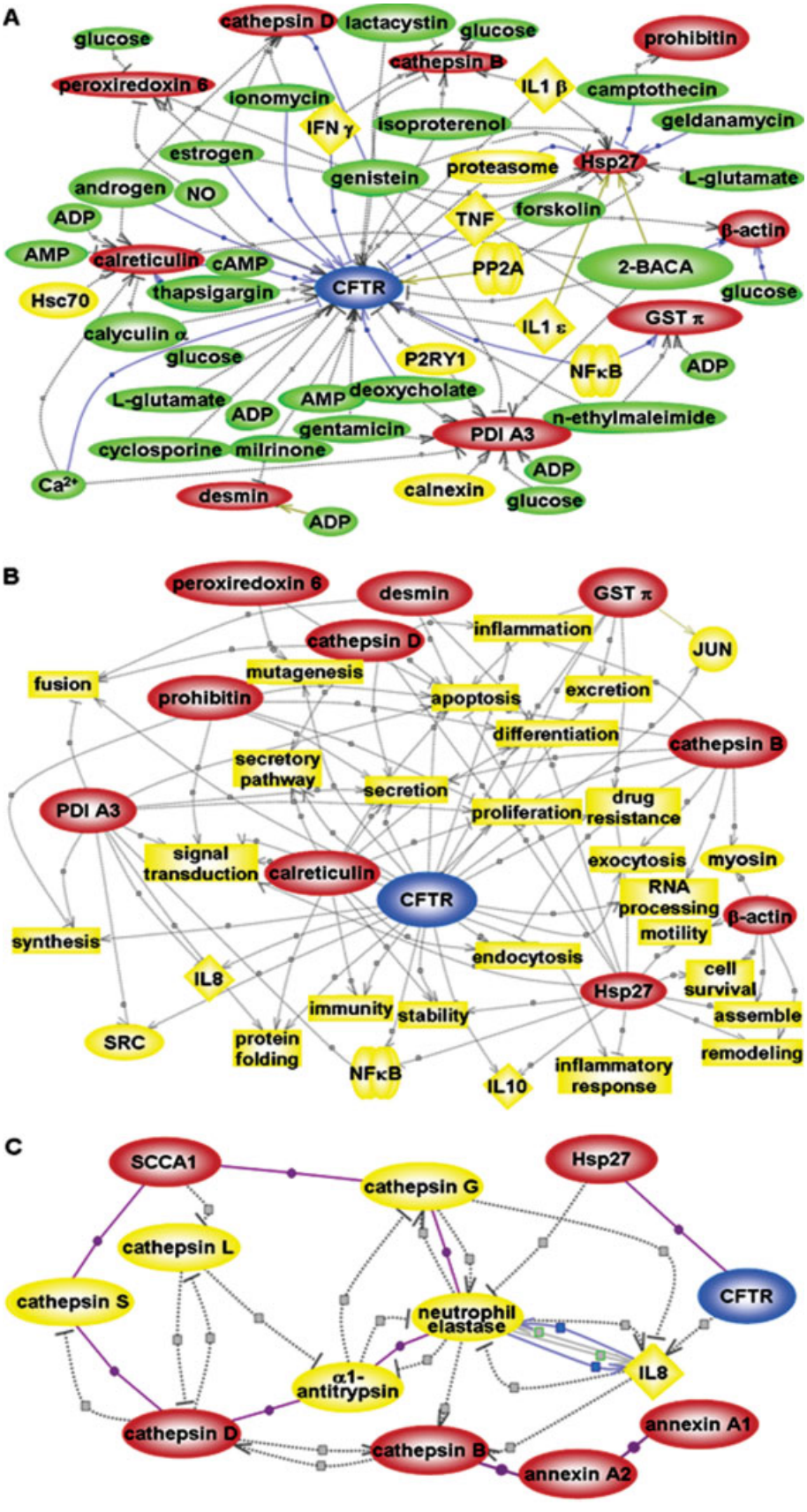

Figure 4. Correlation pathways between CFTR and the proteins that were found to be differentially expressed in CF nasal cells, in comparison to nasal cells from non-CF controls. These pathways were built using the software PathwayAssist version 3.0 (see Section 2). (A) Regulatory pathway, showing the common regulators (in green) of those proteins; (B) biological function pathway, showing common target cellular processes (yellow rectangles); (C) pathway of the proteases and respective inhibitors involved in inflammation. Proteins identified in the 2-DE nasal cell proteome are indicated in red (some of them were not integrated in these pathways as they could not be related to any of the others); proteins added by the software to complete the pathway are shown in yellow. 2-BACA, 2-benzoxazolecarboxylic acid; CFTR; CF-transmembrane conductance regulator; GSTπ, Glutathione-S-transferase π; IFN γ, interferon γ; IL, interleukin; JUN, proto-oncogene c-jun; NFκB, nuclear factor κB; NO, nitric oxide; PDI A3, protein disulfide isomerase A3; PP2A, protein phosphatase 2A; P2RY1, purinergic receptor P2Y; SRC, proto-oncogene tyrosine-protein kinase Src; TNF, tumor necrosis factor. Symbols: positive regulation; negative regulation; regulation of protein expression; molecular transport; protein modification; binding; protein complexes; interleukins.

expression pattern of two lysosome proteases, cathepsins B and D (Table 2, spots 16 and 17), in CF nasal cells in comparison to non-CF. These two enzymes are generally involved in inflammatory processes (Fig. 4C) being, however, regulated in a noncoordinated manner, as indicated by lung tumor studies [15]. Our results support the latter concept by showing that whereas cathepsin B expression is down-regulated in CF patients, cathepsin D is up-regulated.

An enhanced activity of cathepsin B was reported to occur in the sputum of inflamed CF-airways [16] and also in the serum of CF patients [17]. However, in our studies cathepsin B precursor appears to be significantly less expressed in CF than in non-CF nasal cells. This is not entirely surprising as these CF patients were not in an acute phase of inflammation at the time of sample collection. Additionally, they were under constant Pulmozyme treatment. This DNase renders the mucus more fluid, by hydrolyzing the DNA released by the neutrophils, thus reducing mucus obstruction, and breaking the inflammatory cycle. In contrast and probably because it is regulated by different factors, cathepsin D precursor is more abundant in CF nasal cells (Fig. 4A and B).

PLUNC seems to play an important role in host defense against bacteria [18] and in airway inflammatory response after exposure to irritants [19]. This protein (spot 46) was found to be up-regulated in CF cells, although not significantly (Fig. 2A and B; Table 2). Our results are therefore in agreement with the previously described higher expression levels of PLUNC in chronic bronchitis, emphysema, [20], and after invasive surgery [12].

It was reported that CF patients have significantly higher levels of serum IgG antibodies against Hsp27, Hsp 60 [21], and Hsp90 [22] than non-CF controls. However, it is not known whether this results from induced levels of human or bacterial Hsps. Our results indicate that, at least, Hsp27 (spot 30b) is expressed in significantly lower levels in CF patients *versus* controls (Table 2). Hsp27 is an ATP-independent chaperone that functions as an inhibitor of nuclear factor κB (NFκB) pathway [23]. Therefore, the reduced levels of this negative regulator might explain the increased activation of the NFκB and overproduction of interleukin 8 (IL8) reported to occur in CF bronchial gland cells and CF lungs ([24, 25], respectively).

Annexin A1 is a calcium-dependent phospholipid-binding protein that has been implicated in regulating phospholipid vesicle aggregation, mediating inflammatory response [26]. It was reported that the intact form of this protein is not detected in bronchoalveolar lavage from CF patients, in contrast to non-CF controls [27]. Probably due to the fact that CF patients included in this study were not in an exacerbated condition, we did not detect any difference between expression levels of CF and non-CF individuals for either of the two annexin A1 isoforms (spots 7a and b, Fig. 2A *vs.* B; Table 1).

4.2 Oxidative stress

Due to the chronic inflammation, reactive oxygen species (ROS) levels are described to be highly increased in CF (reviewed in [28]), as antioxidant defenses are scarce for the amount of ROS produced. This continuous oxidative stress is therefore responsible for the substantial tissue damage that occurs in the airway surface of CF patients [29]. Also, contributing to this situation is the reduction in the levels of glutathione (in its reduced form) that was reported to occur in the CF patients' airway surface fluid [30], possibly because its efflux is also regulated through CFTR [31]. Besides its antioxidant role, the reduced form of glutathione also contributes to the detoxification of oxidative stress products, as it conjugates to many hydrophobic and electrophilic compounds, through a reaction that is catalyzed by GSTπ [32]. Recently, a polymorphism of the *GSTM3* gene, another GST family member, was associated to CF severity [33]. Consistent with the idea that the GST family may contribute to CF phenotype, we found the expression of GSTπ (spot 27) to be significantly lower in the 2-DE protein profile of CF nasal cells (Table 2).

Peroxiredoxin 6 (spot 45b) was also found to be less abundant in cells from CF patients. This enzyme is a glutathione-dependent peroxidase which is also involved in lung oxidative stress defence. In fact, in rat kidney the levels of its mRNA are induced after intraperitoneal administration of chloroform [34]. Thus, in addition to the previously reported decrease in the CFTR-associated glutathione efflux levels [31], we determined that CF patients also have lower levels of both GSTπ and peroxiredoxin six enzymes in their nasal cells, which therefore results in less protection against the detrimental effects of oxidative stress. Further supporting this idea, Hsp27 (spot 30b), which as mentioned above is less abundant in CF nasal cells, was also described as being one of the major protectors against the oxidative stress caused by chronic inflammation, both through its ability to raise the intracellular glutathione levels [35] and through its chaperone activity on the enzymes involved in detoxification of ROS, such as glutathione transferases and reductases [36].

4.3 Cytoskeleton organization

The integrity and function of epithelial cells are totally dependent on their polarization status, in which the cytoskeleton actin microfilaments play a crucial role [37]. Moreover, it was demonstrated that for cAMP-dependent activation of CFTR, a proper actin organization is required [38]. However, both chronic inflammation and oxidative stress originate from extensive airway tissue injury, inducing major structural changes in the airway wall cells of CF patients. Certainly contributing to these changes are alterations in cytoskeleton organization of such cells. Supporting these observations, we observed that β-actin (spot 12), desmin (spot 19), and tropomyosin α3 chain (spot 55) are expressed in higher levels in nasal cells from CF patients than in non-CF controls

(Table 2). Interestingly, two other isoforms of tropomyosin (Tm5a and Tm5b) were previously implicated in the regulation of CFTR membrane traffic [39], as decreased expression of these proteins leads to an increase in the apical surface targeting of CFTR.

Besides its role in inflammation and oxidative stress protection, Hsp27 seems to be involved in cytoskeleton organization, through the control of actin polymerization/depolymerization processes [40]. Thus, the decrease in the levels of this chaperone in CF nasal cells most probably contributes to the remodeling of this tissue.

4.4 Molecular chaperones

Calreticulin is a fairly well described calcium (Ca^{2+})-dependent molecular chaperone, which also plays a role in the regulation of gene expression and in cell adhesion [41]. Furthermore, it is well established that during infection-associated inflammation, this protein can be released from neutrophils [42]. Here, we determined that calreticulin (spot 15) is expressed in lower levels in nasal cells from CF patients than in controls, although this difference was found to be of no statistical significance.

Protein disulfide isomerases constitute a family of ER enzymes that catalyzes formation of native disulfide bond(s) of nascent peptides and also act as molecular chaperones assisting in protein folding [43, 44]. The expression of one isoform of this family, PDI A3 (spot 49b), was found to be upregulated in nasal cells from CF patients in comparison to non-CF controls (Fig. 2A and B; Table 2). The association of this observation with the fact that all patients analyzed here express F508del-CFTR, a misfolded protein that is mostly retained in the ER for degradation, remains to be investigated.

4.5 Metabolic functions

As mentioned above, the protein 2-DE maps that we obtained here evidenced that there are other proteins differentially expressed between nasal cells from CF patients and non-CF controls. One of them is the 30 kDa subunit of NADH-ubiquinone oxidoreductase (spot 42) that is downregulated in these patients. Interestingly, it was early reported that a decrease occurs in the apparent binding substrate constant (K_m) of this enzyme in skin fibroblast [45] and blood cells from CF patients [46]. Prohibitins, a conserved family of proteins in eukaryotic cells with proposed functions in cell cycle progression, senescence, and apoptosis, have been recently reported as a novel type of membrane-associated chaperones involved in the assembly of mitochondrial respiratory chain complexes [47]. The lower abundance of the mitochondrial proteins ATP synthase D chain (spot 11), NADH-ubiquinone oxidoreductase (spot 42), and prohibitin (spot 47) in CF nasal cells is consistent with the old notion that mitochondrial metabolism is not fully functional in CF tissues.

We also observed that both alcohol dehydrogenase ($NADP^+$) (spot 4) and dihydrodiol dehydrogenase 2 (spot 20) occur in significantly lower levels in CF nasal cells 2-DE maps. As members of the aldo/keto reductases (AKRs) superfamily, these two $NADP(H^+)$-dependent oxidoreductases catalyze the conversion of aldehydes and ketones to alcohols during the metabolism of drugs and xenobiotics [48].

Further studies will be required to understand the relationship between the changes observed here in expression levels of these proteins and the CF respiratory disease.

5 Conclusions

The proteome is a highly dynamic entity, being constantly altered during the cell lifetime. Additionally, changes in protein levels do not result exclusively from up- or down-regulation of their respective gene expression. It could result from PTMs and different processing and/or turnover rates. Therefore, the independent validation of each protein identified here as a contributor to CF pathophysiology will be necessary. Notwithstanding, at least some of those proteins might be associated with respiratory diseases and ultimately with CF pathology. Once validated, CF biomarkers could also be potential targets for the development of new CF therapies.

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