A monoclonal antibody prevents aggregation of the NBD1 domain of the cystic fibrosis transmembrane conductance regulator

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The homozygous deletion of the phenylalanine at position 508 (ΔPhe508) in the first nucleotide-binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR) is the most common CF-causing genetic defect. It has been proposed that the propensity of NBD1 to aggregate may lead to a lower display of the CFTR chloride channel to the cell membrane and to the disease, thus opening an avenue for the pharmacological development of CFTR folding correctors. Here, we show that a human monoclonal antibody fragment specific to the folded conformation of NBD1 inhibits the aggregation of NBD1 *in vitro*. However, in contrast to the previously published observations, we proved experimentally that NBD1 of wild-type and ΔPhe508 version of CFTR display comparable propensities to aggregate *in vitro* and that the corresponding full-length CFTR protein reaches the cell membrane with comparable efficiency in mammalian cell expression systems. On the basis of our results, the 'folding defect' hypothesis seems unlikely to represent the causal mechanism for the pathogenesis of CF. A solid understanding of how the ΔPhe508 deletion leads to the disease represents an absolute requirement for the development of effective drugs against CF.
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Introduction

Certain mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an integral membrane protein, can cause CF, the most common hereditary monogenic disease which affects the entire body, leading to progressive disability and early death (Kerem *et al*., 1997). CF commonly manifests with pancreatic insufficiency, elevated sweat chloride concentration and male infertility. The most striking CF phenotype is seen in airways where chronic pulmonary infections arise from bacterial pathogens. The homozygous deletion of the phenylalanine at position 508 (ΔPhe508) in the first nucleotide binding domain (NBD1) of CFTR is the most common CF-causing mutant, accounting for ∼70% of the CF patients worldwide, yet its frequency varies between ethnic groups, e.g. 82% of CF patients have

homozygous ΔPhe508 in Denmark versus 32% in Turkey (Ratjen and Doring, 2003).

The monogenic nature of the genetic defects in CF makes this disease an attractive candidate for somatic gene therapy, but limited extent and duration of the corrections as well as concerns about the safety of currently available delivery systems have prevented gene therapy from being curative (Ziady and Davis, 2006). Transgene expression should be conserved over the lifetime of the patient. Repeated administrations of both viral and non-viral vectors are hampered by host immunity and low transfection efficiency. Moreover, access of vectors to target cells from the airways is hindered by the thick mucus layer of CF lung. These serious challenges for the gene therapy of CF have stimulated the search for pharmacological agents, an activity which requires a solid understanding of the molecular mechanism by which the ΔPhe508 deletion causes the disease.

Some 20 years after the identification of CFTR mutations as the cause of CF, two alternative theories have been proposed as mechanisms of pathogenesis: the 'folding hypothesis' (according to which CFTR mutations would lead to reduced protein display on the cell membrane) and the 'abnormal gating hypothesis' (according to which mutations lead to abnormal channel function).

The ΔPhe508 deletion has been postulated to give rise to a temperature-sensitive folding defect of the CFTR channel, evidenced by failure of the full-length protein to mature (Denning *et al*., 1992) and by retention in the endoplasmic reticulum and subsequent degradation (Kopito, 1999). The observation that the folding defect of CFTRΔPhe508 could be corrected by growing cells at lower temperature (Denning *et al*., 1992) suggests that molecules capable of stabilizing the folded conformation of CFTR may facilitate the development of drugs which act by increasing folding yields ('correctors') and/or by increasing the specific ion channel activity ('potentiators').

The temperature-dependent folding defect postulated by Denning *et al*. (1992) on the basis of studies of CFTR glycosylation in stably transfected 3T3 fibroblasts is not in complete agreement with experimental evidence of protein expression *in vivo*. First, a careful immunohistochemical analysis of CFTR expression in normal tissues and in CF samples revealed similar levels of protein expression in lung and intestine. However, different levels of CFTR expression were observed in the luminal surface of reabsorptive ducts of sweat glands (Kalin *et al*., 1999). Second, transient transfections of insect cells with CFTR and CFTRΔPhe508 revealed comparable glycosylation patterns and similar expression levels at 27°C. The proteins, when purified and reconstituted *in vitro*, demonstrated comparable activities (Li *et al*., 1993). Moreover, a study performed on immortalized epithelial cells

obtained from healthy donors and CF patients revealed a comparable expression pattern both in terms of protein amount, glycosylation and membrane distribution (Sarkadi *et al.*, 1992).

The folding defect hypothesis is supported by the observation that the ΔPhe508 form of recombinantly expressed NBD1 exhibited a temperature-sensitive tendency to aggregate in *in vitro* refolding experiments, which was more severe compared with the wild-type (wt) protein. Folding yields were measured by diluting a concentrated solution of denatured recombinant NBD1 into a suitable refolding buffer, followed by overnight incubation, centrifugation to remove precipitated protein and fluorescence-based detection of the fraction of soluble NBD1 (Qu and Thomas, 1996; Thibodeau *et al.*, 2005). However, the method did not feature a direct detection of the amount of precipitated protein: a decrease in fluorescence signal could in principle result both from protein precipitation and from the hypofluorescence associated with the denatured form of the protein. The ΔPhe508 form of the recombinant NBD1, containing residues 404–589, was reported to display a slightly reduced folding yield compared with the wt counterpart, both at 2 and 18 μM concentration (Qu and Thomas, 1996). In contrast, the longer version (residues 389–655) of the NBD1 was reported to exhibit a dramatic difference in folding efficiency, with the wt protein being completely folded and soluble at 10°C, whereas the ΔPhe508 form exhibited only 50% folding yield at the same temperature (Thibodeau *et al.*, 2005). According to these experiments, the different *in vitro* refolding behavior of the two versions of the NBD1 proteins would recapitulate the folding defects observed in cellular systems for the full-length CFTR proteins (Denning *et al.*, 1992) and could represent the basis for the high-throughput screening of folding correctors.

The 'abnormal gating hypothesis' mainly relies on experimental data in cell lines (Hwang *et al.*, 1997) and in *Xenopus oocytes* (Treharne *et al.*, 2007), indicating that the ΔPhe508 deletion in CFTR leads to impaired halide transport in model cellular systems. CFTR is a major epithelial ion channel in the apical membrane of the highly active salt absorptive duct of the human sweat gland (Reddy and Quinton, 2006), and patients with CF typically have increased salt concentrations in sweat.

In this article, we report that in our hands the previously published differences in *in vitro* folding yields of wt and ΔPhe508 NBD1 proteins could not be reproduced. In a variety of experimental conditions and using the previously described recombinant versions of the NBD1 (residues 404–589 and 389–655), we observed a comparable temperature and time-dependent profile of protein precipitation for both wt and ΔPhe508 forms. Additionally, both transient and stable transfection experiments performed with vectors encoding the full-length wt and ΔPhe508 forms of CFTR led to comparable levels of channel display on the surface of CHO cells, LM fibroblasts and SP2/0 cells, as revealed by fluorescence-activated cell sorting (FACS) analysis. Interestingly, the folding defects of both wt and ΔPhe508 forms of NBD1 could be corrected in the presence of a monoclonal antibody fragment specific to the native form of the protein. These results indicate that folding correction may be achieved with molecular agents which

stabilize the folded conformation of NBD1, but at the same time underline the need for a critical re-evaluation of the claimed contribution of folding defects to the pathogenesis of CF.

Materials and methods

Protein cloning, expression and purification

Unless stated otherwise, chemicals were purchased from Fluka.

The gene coding for the fragment 404–589 of CFTRwt was PCR amplified from a human cDNA library (Human MTC Panel I, Clontech) with primers Gly-404 (5′-ATAGG ACATATGGGGATTTGGGGAATTATTTGAGAAAGC-3′) and Ser-589 (5′-ATAGGACTCGAGTTAGCTTTCAAATAT TTCTTTTTCTGTTAAAAC-3′) and cloned in pET28 vector (Novagen) using the restriction sites *Nde*I and *Xho*I.

The gene coding for the fragment 389–655 of CFTRwt was PCR amplified from a human cDNA library (Human MTC Panel I, Clontech) with primers Thr-389 (5′-GGG AATTCCATATGACTACAGAAGTAGTGATGGAGAAT-G-3′) and Ala-655 (5′-CCGCTCGAGTTATGCACTAAATT GGTCGAAAGAATC-3′) and cloned in pET28 vector using the restriction sites *Nde*I and *Xho*I. The genes coding for the fragment 404–589 of CFTRwt and 389–655 of CFTRwt were PCR mutated in order to delete the phenylalanine residue at position 508: the deletion was introduced with primers 508bw (5′-GGTGTTTCCTATGATGAATATAGATA C-3′) and 508mut (5′-TCTATATTCATCATAGGAAA CACCGATGATATTTTCTTTAATGGTGCCAG-3′) and cloned in pET28 vector using the restriction sites *Nde*I and *Xho*I. BL21 (DE3) *Escherichia coli* (Invitrogen) was transformed with pET28–NBD1(404–589)wt, pET28–NBD1 (404–589)ΔPhe508, pET28–NBD1(389–655)wt or pET28–NBD1(389–655)ΔPhe508. The corresponding proteins, which contain an initial GSSHHHHHHSSGLVPRGSHM sequence followed by amino acids 404–589 (GFGELFEKA KQNNNNRKTSNGDDSLFFSNFSLLGTPVLKDINFKIER-GQLLAVAGSTGAGKTSLLMMIMGELEPSEGKIKHSGRI-SFCSQFSWIMPGTIKENIIFGVSYDEYRYRSVIKACQLEE DISKFAEKDNIVLGEGGITLSGGQRARISLARAVYKDAD LYLLDSPFGYLDVLTEKEIFES) or by amino acids 389–655 (TTEVVMENVTAFWEEGFGELFEKAKQNNN NRKT SNGDDSLFFSNFSLLGTPVLKDINFKIERGQLLAVAGST-GAGKTSLLMMIMGELEPSEGKIKHSGRISFCSQFSWIMP GTIKENIIFGVSYDEYRYRSVIKACQLEEDISKFAEKDNI-VLGEGGITLSGGQRARISLARAVYKDADLYLLDSPFGY-LDVLTEKEIFESCVCKLMANKTRILVTSKMEHLKKAD-KILILNEGSSYFYGTFSELQNLQPDFSSKLMGCDSFDQF-SA), were expressed as described below.

After transformation of BL21 (DE3) cells, colonies harboring NBD1 constructs were inoculated in 100 ml 2 × YT medium (Q-BIOgene) containing 30 μg/ml kanamycin (Applichem) and 1% (w/v) glucose (Sigma-Aldrich) and grown overnight at 30°C in a rotary shaker at 200 rpm. A volume of 5 ml from the overnight pre-culture was then inoculated into 1000 ml 2 × YT medium containing 30 μg/ml kanamycin and 0.1% (w/v) glucose and grown at 37°C in a rotary shaker at 200 rpm until the absorbance at 600 nm reached a value between 0.4 and 0.6. Protein expression was induced by the addition of 1 mM IPTG

(Applichem). After 4 h at 37°C in a rotary shaker (200 rpm), the bacterial cells were harvested by centrifugation and resuspended in 6 ml buffer W1 (20 mM Tris, 500 mM NaCl, 6 M Guanidine hydrochloride, 5 mM Imidazole, pH 7.9). After cell lysis by sonication (Vibracell CV33, SONICS), the lysate was centrifuged for 20 min at 47 800 g. The cleared lysate was mixed with 4 ml of Ni^{2+}-NTA slurry (Qiagen) to capture the $6 \times$ His tagged proteins and the mixture was incubated at 4°C for 1 h while shaking on a rotary shaker. The resin was washed first with 20 ml buffer W1, then with buffer W2 (20 mM Tris, 500 mM NaCl, 6 M Guanidine hydrochloride, 20 mM Imidazole, pH 7.9) until the absorbance at 280 nm of the eluate was lower than 0.1. The protein was eluted by the addition of 12 ml buffer E (20 mM Tris, 500 mM NaCl, 6 M Guanidine hydrochloride, 400 mM Imidazole, pH 7.9). After elution, the protein was dialyzed overnight at 4°C against buffer D (20 mM Tris, 6 M Guanidine hydrochloide, 1 mM DTT, pH 8.0). The protein stock was concentrated by centrifugation with Vivaspin tubes (10 000 MWCO PES, Vivascience) and stored at −20°C. SDS−PAGE (Invitrogen) analysis was performed on samples after overnight ethanol precipitation, to avoid the presence of Guanidine hydrochloride.

The correctness of the cloning was confirmed by sequencing using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystem). Furthermore, the proteins were characterized by analyzing their tryptic digests by MALDI-TOF/TOF mass spectrometry. Both proteins were digested overnight with trypsin (Promega). Resulting peptides were measured on a 4700 Proteomic Analyzer (Applied Biosystems) and the spectra were analyzed using the GPS Explorer and the Data Explorer software (Applied Biosystems).

Refolding experiments

Aliquots of purified NBD1wt or NBD1ΔPhe508 stocks were diluted 30-fold with buffer B (100 mM Tris, 400 mM L-arginine, 2 mM EDTA, 1 mM DTT, pH 8.0) to a final protein concentration of 1−20 µM. The refolding step was performed at 4°C using tips, tubes and solutions previously equilibrated at 4°C in order to avoid any unwanted protein precipitation. Samples were incubated at different temperatures, from 4°C to 37°C, and for different time gaps. After incubation, samples were centrifuged for 10 min at 15 700 g and 4°C, to remove insoluble misfolded protein.

Temperature dependence of NBD1 folding: tryptophan fluorescence

In order to study the temperature dependence of folding, NBD1(389−655)wt and NBD1(389−655)ΔPhe508 were refolded, incubated overnight and centrifuged as described above. After centrifugation, tryptophan fluorescence emission spectra of supernatants were collected. Samples were analyzed with the spectrofluorometer LS50b (Perkin Elmer), using 282 nm excitation light and monitoring emission between 300 and 400 nm. The peak value of protein refolded and incubated at 4°C was considered as corresponding to 100% of soluble protein.

Temperature dependence of NBD1 folding: SDS−PAGE

In order to study the temperature dependence of folding, NBD1wt and NBD1ΔPhe508 were refolded, incubated and centrifuged as described above. After centrifugation, pellets and supernatants were separated, pellets were dissolved and supernatants were diluted to the same final volume by adding H_2O and gel loading buffer (0.2 M Tris, 30% glycerol, 230 mM Sodium dodecyl sulfate, 1 mM Bromophenol Blue). Ten microliters of pellet and supernatant samples were analyzed by SDS−PAGE (10% Bis−Tris Gel, Invitrogen).

Antibody selection

Human monoclonal antibodies specific to NBD1ΔPhe508 were isolated by biopanning from the ETH2-Gold antibody phage display library, previously described by our group (Viti et al., 2000; Silacci et al., 2005). Biopanning experiments were performed on immunotubes coated with recombinant NBD1ΔPhe508 following standard procedures (Viti et al., 2000), confirming antigen binding both by ELISA on microtiter plates and by antigen immobilization on a Biacore CM-5 biosensor chip (Biacore). The isolated clones were sequenced using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystem). One of the NBD1-binding scFv fragments ('C2') was tested for its folding stabilizing effect. Temperature-dependent folding experiments were performed as described above, adding equimolar amounts of scFv C2 to the folding buffer just before addition of NBD1. Samples were incubated at different temperatures for increasing time intervals and prepared for SDS−PAGE analysis as described above.

FACS analysis of transfected mammalian cells

CHO-S cells (GIBCO, Invitrogen) were transfected (Gene Pulser, Bio-Rad) with pcDNA3.1 vector containing the coding sequence for either CFTRwt or CFTRΔPhe508 (vectors kindly provided by Prof. Galietta). After electroporation, cells were grown at 37°C for 72 h in CD CHO medium (GIBCO, Invitrogen) including HT Supplement (GIBCO, Invitrogen).

LM fibroblasts (ATCC, CCL-1.2) were transfected with pcDNA3.1 vector containing the coding sequence for either CFTRwt or CFTRΔPhe508, with the transfection reagent Lipofectamine 2000 (Invitrogen). After transfection, cells were grown at 37°C for 48 h in DMEM medium (GIBCO, Invitrogen) including fetal bovine serum (FCS, GIBCO, Invitrogen) and antibiotic−antimycotic (GIBCO, Invitrogen).

SP2/0 cells (ATCC, CRL-1581) were transfected (Nucleofector, Amaxa) with pcDNA3.1 vector containing the coding sequence for either CFTRwt or CFTRΔPhe508. After transfection, cells were grown at 37°C for 48 h in CD Hybridoma medium (GIBCO, Invitrogen) including L-Glutamine (GIBCO, Invitrogen) and antibiotic−antimycotic (GIBCO, Invitrogen).

Cells were harvested and diluted in PBS containing 1% FCS (FPBS) to a 5×10^6 cell/ml concentration: 200 µl of this cell solution were used for each single staining. Cells were spun at 292 g for 5 min, the supernatant was discarded and 100 µl of a 2 µl/ml solution of the primary antibody (mouse monoclonal [CF3] to CFTR, Ab2784, Abcam) was added to the cells. Cells were incubated for 20 min at 4°C. After incubation, cells were spun at 292 g for 5 min, the supernatant was discarded and 100 µl of a 50 µl/ml solution of the secondary antibody (R-Phycoerithrin-Conjugated Rat Anti-Mouse IgM Monoclonal Antibody, BD Pharmingen) was added to the cells. Cells were incubated for 20 min at 4°C in the dark. After incubation, cells were spun at 292 g
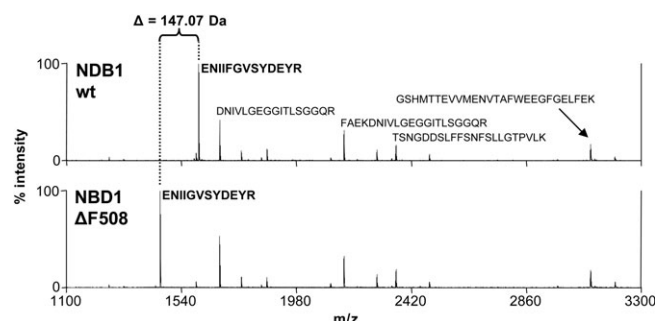
Fig. 1. Mass spectra of tryptic peptides of wt and ΔPhe508 NDB1(389–655). The two spectra show comparable peak profiles. Only two peaks are different, with a mass difference corresponding to the monoisotopic mass of phenylalanine. MALDI-TOF/TOF analyses of these two peaks led to identification of the peptide ENIIFGVSYDEYR in the wt spectrum and the peptide ENIIGVSYDEYR in the ΔPhe508 spectrum. The sequences of the main peptide peaks are indicated.

for 5 min, the supernatant was discarded and cells were washed by adding 200 µl of FPBS. Cells were immediately spun at 292 g for 5 min, the supernatant was discarded and cells were finally resuspended in 100 µl of FPBS. Cell samples were stored at 4°C in the dark before analysis with a BD FACSCanto Flow Cytometer (BD Biosciences).

Results

In vitro *refolding experiments with recombinant NBD1wt and NBD1ΔPhe508 proteins*

We cloned and expressed in *E.coli* the wt and ΔPhe508 form of the NBD1 domain containing residues 404–589 and 389–655, carrying a 6 × His-tag at the N-terminus of the protein. The putative amino acid sequence for all four proteins variants was identical to the one described in previous publications (Qu and Thomas, 1996; Thibodeau *et al.*, 2005). Figure 1 shows a portion of the mass-spectrometric characterization performed on the recombinant 389–655 versions

of the NBD1 domain. A protein coverage of 78.7% for both proteins could be confirmed with tryptic peptides, including an unambiguous detection of N- and C-terminal peptides and a detected mass difference of 147.07 Da for the tryptic peptides corresponding to residues 136–148 of NBD1wt and to residues 136–147 of NBD1ΔPhe508.

In order to shed light on the mechanism of incomplete folding yields (i.e. low fluorescence values could be due to either protein precipitation during the overnight incubation and/or lower fluorescence intensities for soluble protein in the unfolded conformation), we performed an SDS–PAGE analysis of protein refolding, separating soluble and insoluble protein by centrifugation. The short version of recombinant NBD1, containing residues 404–589, displayed to be soluble when incubated at 4°C (Fig. 2). Incubating the NBD1(404–589) protein samples at 22°C resulted in growing amounts of insoluble protein at increasing incubation time (Fig. 2). These data demonstrate how increasing incubation time and temperature will result in increment of insoluble protein. However, the wt and mutant versions of the NBD1(404–589) failed to display any differences in aggregation rate.

Using the same experimental procedure, we then analyzed the folding behavior of the 389–655 versions of the NBD1 domain. Figure 3 shows a representative analysis of several refolding experiments performed at different times and temperatures. As for the shorter version of the protein, we could confirm that at increasing temperatures and incubation times, NBD1(389–655) would yield increasing amounts of insoluble protein, as previously described in other works (Qu and Thomas, 1996; Thibodeau *et al.*, 2005). However, in our hands, no significant difference could be detected between the NBD1(389–655)wt and the corresponding NBD1(389–655)ΔPhe508 mutant, in clear contrast with the data reported by Thibodeau and coworkers. Similarly, we did not observe differences in folding yield between the two forms of the NBD1(389–655) using fluorescence detection methodologies (Fig. 4).
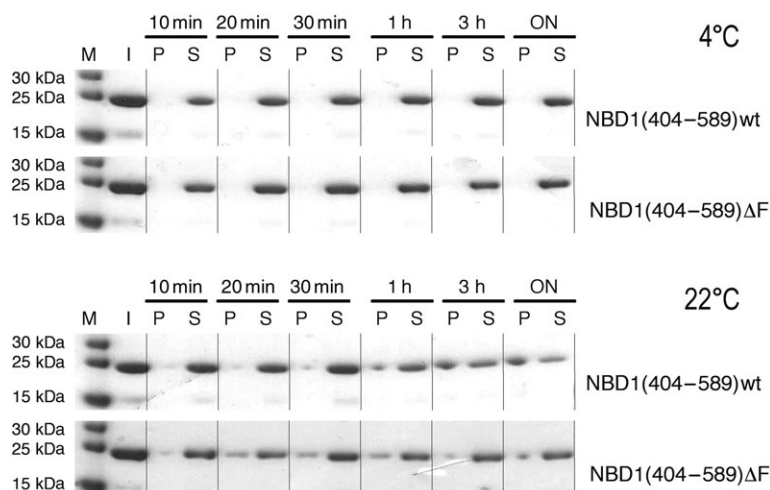

Fig. 2. SDS–PAGE analysis of NBD1 folding efficiency. NBD1(404–589)wt and NBD1(404–589)ΔPhe508 proteins were refolded by dilution to roughly 20 µM final concentration as described in the text (see 'Materials and methods'). Samples were incubated for increasing times at the indicated temperatures and, after centrifugation, pellets (P) and supernatants (S) were separated and analyzed. Input (I) corresponds to not centrifuged samples. Each time point includes 10 min centrifugation (i.e. 30 min stands for 20 min of incubation plus 10 min of centrifugation). The overnight (ON) incubation corresponds to 16 h. NBD1(404–589)wt and NBD1(404–589)ΔPhe508 proteins were soluble after overnight incubation at 4°C. Both proteins showed comparable amounts of pellet after the same incubation time at 22°C.
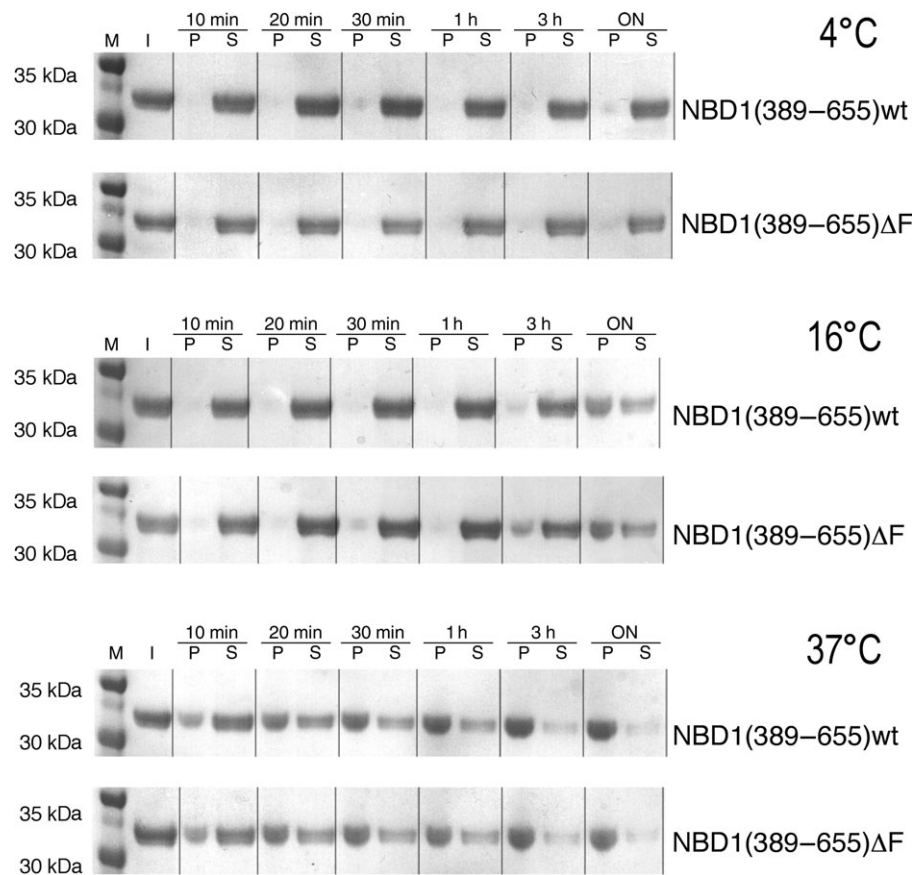
Fig. 3. SDS–PAGE analysis of NBD1 folding efficiency. NBD1(389–655)wt and NBD1(389–655)ΔPhe508 proteins were refolded by dilution to 8 μM final concentration as described in the text (see 'Materials and methods'). Samples were incubated for increasing times at the indicated temperatures and, after centrifugation, pellets (P) and supernatants (S) were separated and analyzed. Input (I) corresponds to not centrifuged samples. Each time point includes 10 min centrifugation (i.e. 30 min stands for 20 min of incubation plus 10 min of centrifugation). The overnight (ON) incubation corresponds to 16 h. NBD1(389–655)wt and NBD1(389–655)ΔPhe508 proteins were soluble after overnight incubation at 4°C. Both proteins showed comparable amounts of pellet after the same incubation time at 16°C and 37°C.

Refolding of NBD1 in presence of a human monoclonal antibody specific to NBD1

Attempts to increase the recovery of soluble folded NBD1 proteins at higher temperatures failed (data not shown) in the presence of 1 mM ATP, 1 mM TNP–ATP (an ATP-analogue) and 1 mM concentrations of drugs such as BayK-8644, Curcumin and Genistein, which are under consideration for CF therapy (Weinreich *et al.*, 1997; Davis and Drumm, 2004; Pedemonte *et al.*, 2005).

In order to determine whether a binder, endowed with sufficient affinity and specificity for the NBD1 domain, could stabilize the folded conformation of this protein and reduce precipitation, we generated human monoclonal antibody fragments in scFv format from a large synthetic phage display library recently cloned in our group (Silacci *et al.*, 2005). One of the resulting monoclonal antibodies (clone C2; Table I) exhibited a comparable binding affinity to both NBD1wt and NBD1ΔPhe508 and was thus used as additive in refolding experiments. The scFv(C2) exhibited a substantial improvement in the recovery of soluble NBD1(389–655) domain, when analyzing the NBD1 refolding experiment by SDS–PAGE (Fig. 5), indicating that NBD1-binding molecules may indeed help stabilize the soluble, folded conformation of this protein.
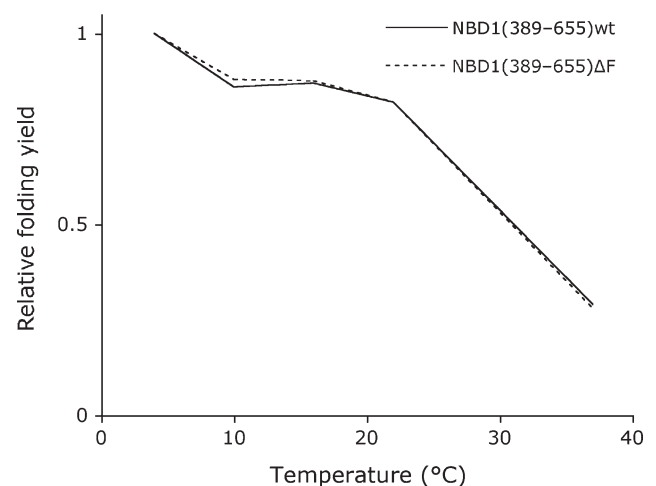

Fig. 4. NBD1 folding efficiency as a function of folding temperature. NBD1(389–655)wt and NBD1(389–655)ΔPhe508 proteins were refolded by dilution to 1 μM final concentration as described in the text (see 'Materials and methods'). Samples were incubated overnight at the indicated temperatures, and after centrifugation, fluorescence emission spectra of the supernatants were collected. The relative folding yield was determined as the fraction of tryptophan fluorescence emission intensity. Samples were excited at 282 nm and emission spectra were collected from 300 to 400 nm. The peak value detected on samples incubated at 4°C was considered as corresponding to 100% of soluble protein.

Heavy chain	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYADSV KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK**MRLGL**FDYWGQGTLVTVSS
Linker	*GGGGSGGGGSGGGG*
Light chain	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGS GSGTDFTLTISRLEPEDFAVYYCQQ**RGDVPP**TFGQGTKVEIKAAAE<u>QKLISEEDL</u>NGAA-

The amino acid sequence of the human monoclonal antibody C2 selected against NBD1 is represented in the table. The scFv is composed of a heavy chain, a linker (indicated in italic) and a light chain; the light chain includes a myc tag that is underlined in the table. The randomized CDR3 regions in the synthetic antibody library used for antibody selection (Silacci *et al.*, 2005) are displayed in bold.

Cell transfection with vectors coding for CFTRwt and CFTRΔPhe508

Puzzled by the discrepancy between the results of our *in vitro* refolding experiments with the NBD1 domain and the previously published data, we decided to critically evaluate the levels of membrane expression of CFTR for the wt and ΔPhe508 version of the protein in a model cellular system. To this aim, we transfected CHO cells, LM fibroblasts and SP2/0 cells with vectors coding either for the wt or for the ΔPhe508 version of CFTR. The transfections of the different cell lines were performed in parallel, using identical amounts of plasmids and number of cells. After transfection, cells were cultured at 37°C. The amount of CFTR protein displayed at the plasma membrane of both transiently and stably transfected cells was detected by FACS using the monoclonal antibody Ab2784 (Abcam), which recognizes an extracellular portion of the CFTR protein (Walker *et al.*, 1995). The analysis of transiently transfected CHO-S cells shows a similar increase of FACS signal for both the wt and the ΔPhe508 version of the protein, compared with the untransfected controls (Fig. 6a). Similar results were obtained when analyzing transiently transfected LM fibroblasts (Fig. 6b) and SP2/0 cells (Fig. 6c), which also revealed comparable levels of wt and ΔPhe508 protein display on the cell membrane.

Discussion

Almost 20 years after the discovery that the ΔPhe508 deletion in CFTR is the most frequent cause of CF (Riordan *et al.*, 1989; Rommens *et al.*, 1989), the mechanisms for the pathogenesis of the disease are still poorly understood. The results of this article on one hand show for the first time that molecular correctors can be identified which rescue folding defects of NBD1 *in vitro*, but at the same time cast doubts about the validity of the 'folding defect' hypothesis for the pathogenesis of CF. Some of the previously published
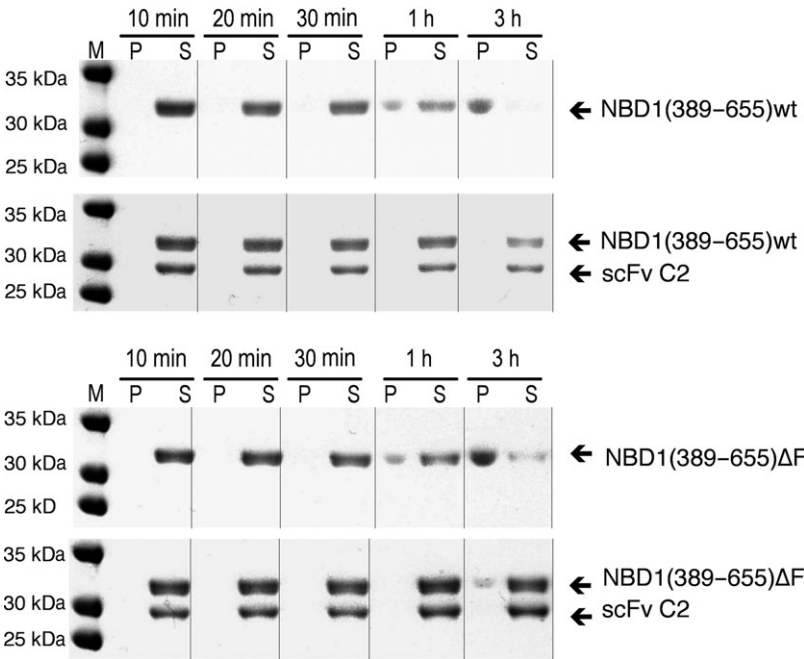

Fig. 5. SDS−PAGE analysis of NBD1 folding efficiency in the presence of scFv C2. NBD1(389−655)wt and NBD1(389−655)ΔPhe508 proteins were refolded by dilution in folding buffer, with or without equimolar amounts of scFv C2, to 8 μM final concentration as described in the text (see 'Materials and methods'). The NBD1-binding scFv C2 antibody fragment runs at ∼27 kDa. Samples were incubated for increasing times at 25°C and, after centrifugation, pellets (P) and supernatants (S) were separated and analyzed. After 1 and 3 h of incubation at 25°C, NBD1(389−655)wt and NBD1(389−655)ΔPhe508 proteins refolded in the presence of scFv C2 and remained soluble, whereas the corresponding samples incubated in folding buffer without scFv C2 were found in the pellet.
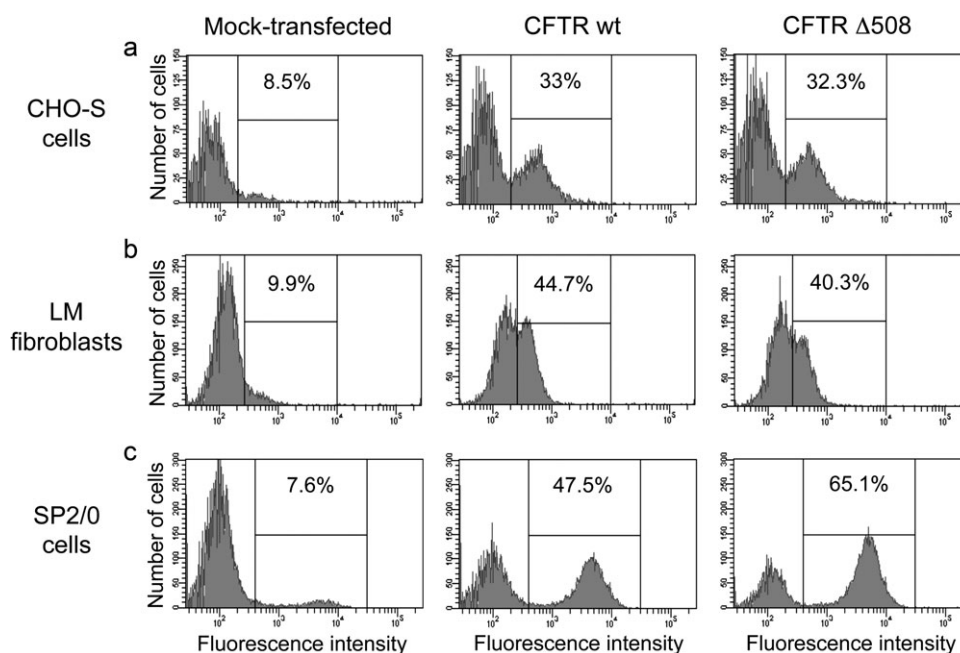
Fig. 6. FACS analysis of CHO-S cells (**a**), LM fibroblasts (**b**) and SP2/0 cells (**c**). The negative controls are shown on the left panels, the cells transfected with pcDNA3.1–CFTRwt vector in the central panels and with pcDNA3.1–CFTRΔPhe508 vector in the right panels. Each sample was incubated both with primary and secondary antibodies (see 'Materials and methods'). The percentage of cell population giving a fluorescence signal corresponding to detection of CFTR is indicated in each panel.

reports on differential CFTR expression relied on expression studies with monoclonal cell lines (Egan *et al.*, 2004). It is well established that cells stably transfected with a given vector typically yield monoclonal cell lines which may differ in protein expression by several orders of magnitude (Newman and Mann, 2007).

It has been proposed that the ΔPhe508 deletion in CFTR leads to impaired halide transport in model cellular systems both using stable transfections for CFTR expression (Hwang *et al.*, 1997) and, importantly, cRNA injection in *Xenopus* oocytes (Treharne *et al.*, 2007). However, we strongly feel that differences in ion transport efficiency should be assessed in the context of the patterns of CFTR expression in different tissues and their pathophysiological implications (Kalin *et al.*, 1999).

One of the most attractive areas of modern CF research focuses on the characterization of the CF 'interactome' (i.e. the set of proteins which may interact directly or indirectly with CFTR) (Ollero *et al.*, 2006; Wang *et al.*, 2006; Treharne *et al.*, 2007). It has recently been suggested that the ΔPhe508 deletion impairs CK2-mediated binding to NBD1 and the phosphorylation of Ser511 within the domain (Treharne *et al.*, 2007), with profound consequences on CFTR channel gating. Mutation of Ser511 within CFTR phenocopies the effect of the ΔPhe508 deletion. If these results are confirmed, it appears that pharmacological research activities should ideally be focused on the identification of selective channel potentiators. In turn, these activities will most likely rely on the identification of specific CFTR-binding molecules.

The finding that the folding defects of both wt and ΔPhe508 forms of NBD1 could be corrected in the presence of a monoclonal antibody fragment specific to the native form of the protein gives hope that NBD1-binding molecules may help stabilize the soluble, folded conformation of this protein. Such a concept has previously been demonstrated for

antibody and peptides molecules recognizing mutant, destabilized p53 transcription factor in the field of cancer research (Issaeva *et al.*, 2003; Weisbart *et al.*, 2004). From a chemical point of view, the 'minimization' of protein-based binders (Braisted and Wells, 1996) or the functional antibody replacement with small organic molecules (Melkko *et al.*, 2007) both represent formidable challenges.

Moreover, the strategy of using small molecules that stabilize a mutant protein and consequently restore trafficking and activity, an approach called molecular chaperoning, has been successfully applied to the lysosomal enzyme acid β-glucosidase (GCase, Lieberman *et al.*, 2007). Mutations in the GCase protein destabilize the native conformation of the enzyme leading to the Gaucher disease. The ability to stabilize the right protein conformation may be the hallmark of a molecule with therapeutic effect.

In summary, we have generated a monoclonal antibody fragment which prevents the aggregation of the NBD1 domain of both wt and ΔPhe508 versions of CFTR. At the same time, we have provided evidence for comparable levels of expression of the full-length CFTR protein in mammalian cells. This observation weakens the 'folding defect' hypothesis, which is still frequently invoked as the causal mechanism of CF (Wang *et al.*, 2006). However, CFTR mutations do lead to abnormal protein expression only in certain tissues, most notably the sweat gland ducts. The reasons for these tissue-specific defects of protein production are not known at present. A better understanding of the molecular mechanisms for the pathogenesis of CF is urgently needed, in order to provide a rational basis for the development of effective drugs for the pharmacological treatment of this disease.

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