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Evidence for alteration of calpain/calpastatin system in PBMC of cystic fibrosis patients

Monica Averna [a], Roberto Stifanese [a], Roberta De Tullio [a], Laura Minicucci [b], Federico Cresta [b], Serena Palena [a], Franca Salamino [a], Sandro Pontremoli [a], Edon Melloni [a,*]

[a] Department of Experimental Medicine (DIMES)-Biochemistry Section, and Center of Excellence for Biomedical Research (CEBR), University of Genoa, Viale Benedetto XV, 1–16132 Genoa, Italy
[b] Cystic Fibrosis Center, Pediatric Department Institute G.Gaslini, Genoa, Italy

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ABSTRACT

We are here reporting that in peripheral blood mononuclear cells (PBMC) of patients homozygous for F508del-CFTR the calpain–calpastatin system undergoes a profound alteration. In fact, calpain basal activity, almost undetectable in control PBMC, becomes measurable at a significant extent in cells from cystic fibrosis (CF) patients, also due to a 40–60% decrease in both calpastatin protein and inhibitory activity. Constitutive protease activation in CF patients' cells induces a large accumulation of the mutated cystic fibrosis transmembrane conductance regulator (CFTR) in the 100 kD + 70 kD split forms as well as a degradation of proteins associated to the CFTR complex. Specifically, the scaffolding protein Na^+/H^+ exchanger 3 regulatory factor-1 (NHERF-1) is converted in two distinct fragments showing masses of 35 kD and 20 kD, being however the latter form the most represented one, thereby indicating that in CF-PBMC the CFTR complex undergoes a large disorganization. In conclusion, our observations are providing new information on the role of calpain in the regulation of plasma membrane ion conductance and provide additional evidence on the transition of this protease activity from a physiological to a pathological function.

1. Introduction

It is clearly emerging that transduction of biological signals is mediated by the dynamic assembly of signalling complexes organized on the inner surface of plasma membranes by transmembrane ion channels [1,2]. A number of these complexes possess on its cytosolic region a PDZ-binding motif which triggers the onset of these protein clusters [3–5]. For instance, this association has been indicated in the case of N-methyl-D-aspartate receptor (NMDAR) and cystic fibrosis transmembrane conductance regulator (CFTR), being both involved in the formation of signalling complexes through their PDZ-binding motif [6–10]. These functional clusters are generated by the association to the PDZ-binding structures of scaffolding proteins, such as PSD95 or EBP50, that contain multiple PDZ domains. Thus the organization of the functional complexes is further extended, with the inclusion of chaperones and enzymes promoting selective signalling cascades. CFTR is assembled in a functional multiprotein complex in which the scaffolding protein NHERF-1 exerts a central role in recruiting different cytoskeletal proteins, such as ezrin, and cAMP-dependent protein kinase (PKA) required for the channel activation [11]. These channels and some of their protein partners can be digested by calpain. Specifically, it has been established that NMDAR subunits are cleaved by calpain [12] and, more recently [13], we have demonstrated that mature CFTR is cleaved by calpain in two discrete fragments having a mass of 100 kD and 70 kD respectively. These fragments remain associated each other into the membrane moiety and are rapidly internalized in endosomes. These sequential events may represent the initial step of CFTR turnover, subsequently completed by lysosomal proteases. We have also observed that F508del-CFTR is more susceptible to calpain proteolysis due to a decreased affinity for HSP90, resulting in a much less efficient protection by this chaperone [14]. In PBMC from CF patients (CF-PBMC), CFTR proteolysis is enhanced, suggesting an overactivation of calpain that finally leads to the almost complete absence of mature CFTR at plasma membrane [14,15].

We are now reporting that in CF-PBMC an unbalance in the calpain/calpastatin system is responsible for overactivation of calpain which in turn degrades NHERF-1 and ezrin modifying also their cellular distribution. NHERF-1 digestion has been confirmed both in reconstructed systems and isolated cells.

Our observations provide additional evidence on the transition of calpain from a physiological to a pathological role, a condition inducing the uncontrolled digestion of these protein complexes.

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; NHERF, Na^+/H^+ exchanger 3 regulatory factor; PBMC, peripheral blood mononuclear cells.

* Corresponding author. Tel.: +39 010 3538128; fax: +39 010 518343.
E-mail address: melloni@unige.it (E. Melloni).

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2. Materials and methods

2.1. Materials

Leupeptin, calcium ionophore A23187 and C.I.1 (calpain inhibitor 1) were purchased from Sigma-Aldrich. 4-(2-aminoethyl) benzene-sulfonylfluoride (AEBSF) was obtained from Calbiochem. ECL ADVANCE® Detection System was obtained from GE Healthcare. t-BOC (t-butoxycarbonyl)-Leu-Met-CMAC (7-amino-4-chlorometylcoumarin), a fluorogenic calpain substrate was purchased from Molecular Probes (Invitrogen). Ficoll-Pacque Plus was obtained from GE Healthcare. Human erythrocyte calpain was isolated and assayed as reported in [16]. One unit of calpain activity is defined as the amount that releases 1 nMol/h of free a-amino groups under the specified conditions [17].

Anti-NHERF-1 (H-100) polyclonal antibody was purchased from Santa Cruz biotechnology, inc. Anti-CFTR monoclonal antibody (clone M3A7) was purchased from Millipore. Anti-CFTR polyclonal antibody was purchased from Cell Signaling. Anti-ezrin monoclonal antibody (18/Ezrin) was purchased from BD Transduction Laboratories™. Monoclonal anti-μ-calpain (calpain I, subunit p80) clone 15C10 was obtained from Sigma-Aldrich. Calpastatin was detected with the monoclonal antibody 35.23 [18].

2.2. Donor subjects and sample collection

12 CF patients homozygous for F508del-CFTR mutation, 3 obligates heterozygotes (CF patient mothers) and 10 healthy donors were enrolled in the study. CF patients (6 males; mean age: 28, range: 9÷55, mean FEV1% predicted value for height, sex and age 54%, range: 20÷108; 12/12 with pancreatic insufficiency) were regularly followed at the Cystic Fibrosis Center, Pediatric Department Institute G. Gaslini, Genoa, Italy. For every patient and healthy donor, a sample of 6 ml of blood was collected in two 3 ml vacuette® PREMIUM tubes containing 5 mM EDTA.

2.3. Assay of intracellular calpain activity

Calpain activity was detected in intact PBMC following the procedure described in [19]. Briefly, PBMC were collected, washed three times in PBS solution, and incubated for 20 min at 37 °C in buffer A (10 mM Hepes, pH 7.4, 0.14 M NaCl, 5 mM KCl, and 5 mM glucose) containing 50 μM t-Boc-Leu-Met-CMAC, the fluorogenic calpain substrate. Cells were washed with buffer A to remove substrate excess. The pellets were resuspended to give 10^6 cells/ml in buffer A containing 0.1 mM $CaCl_2$. Aliquots (2×10^5 cells) were transferred to 96-well plates and the fluorescence emission was continuously monitored at 37 °C with a Mithras LB940 plate reader (Berthold Technologies) in the absence or presence of 2 μM Ca^{2+}-ionophore A23187. The excitation/emission wavelengths were 355/485 nm respectively.

2.4. Separation and quantification of calpastatin species in human PBMC, following SDS-polyacrylamide gel electrophoresis

Crude extracts obtained from 4×10^6 PBMC from healthy donors (C-PBMC) and CF-PBMC were submitted to SDS-polyacrilamide gel electrophoresis (10%) divided in 10 lanes. Calpastatin species were identified following protein extraction from the gel, as previously described [20]. Calpastatin activity was measured as described [21].

2.5. Confocal microscopy imaging and fluorescence quantification

PBMC (10×10^6) isolated as previously described [22] from control and CF patients were fixed and permeabilized by the Triton/paraformaldehyde method, as described in [23]. Cells were treated with 10 μg/ml CFTR antibody (M3A7) or, alternatively, with 4 μg/ml NHERF-1 antibody diluted in PBS solution, containing 5% (v/v) FBS. After incubation for 3 h at 25 °C, cells were washed three times with PBS solution and treated with 4 μg/ml chicken anti-(mouse IgG) Alexa fluor® 488-conjugate secondary antibody (Molecular Probes) for 1 h. Chromatin was stained by exposing fixed and permeabilized cells to 2 μg/ml propidium iodide for 5 min [24]. Images were collected using a Bio-Rad MRC1024 confocal microscopy, with a 60× Plan Apo objective with numerical aperture 1.4. The excitation/emission wavelengths for propidium iodide-stained chromatin were 488–568/605 nm. Sequential acquisitions were performed to avoid cross-talk between colour channels. The fluorescence intensity in each image was quantified using LaserPix software (Bio-Rad) following the procedure described in [25].

2.6. Isolation of the membrane and solubilized fractions from PBMC

PBMC were washed three times with ice-cold PBS solution. The cells (10×10^6) were lysed in 1 ml buffer A (50 mM sodium borate, 1 mM EDTA, pH 7.5), containing 5 mg leupeptin and 10 mg AEBSF, by three cycles of freezing and thawing followed by sonication (total material). The total particulate material was separated from the supernatant by centrifugation at 100,000 *g* for 10 min at 4 °C and washed three times in buffer A (total membrane fraction). When indicated the membrane fractions were washed in ten volumes (500 μl) of buffer A containing 0.5 M NaCl or 1 M NaCl and centrifuged at 100,000 *g* for 10 min. Supernatants (solubilized material) and pellets (washed membranes) were separately collected. The protein concentration was determined following the method of Lowry.

2.7. Immunoprecipitation and immunoblotting

Total membrane fraction isolated from 10×10^6 PBMC, as described earlier, was solubilized in 500 μl buffer A containing 0.15 M NaCl and 1% Triton® X-100. The mixture, pre-treated with protein G-Sepharose, was incubated with 2 μg of anti-CFTR antibody (clone M3A7) for 15 h at 4 °C. Protein G-Sepharose was then added to the sample which was incubated for an additional 1 h with continuous and gentle shaking. The immunocomplexes were washed three times with buffer A, incubated at 95 °C in Laemmli loading buffer for 10 min and submitted to 12% SDS-PAGE [26]. Proteins were then transferred to nitrocellulose membranes (Bio-Rad) by electroblotting [27] and probed with the anti-NHERF-1 antibody, followed by a peroxidase-conjugated secondary antibody as described in [28]. Alternatively three different amounts (10, 20 and 30 μg) of total, membrane, soluble, solubilized, and washed membranes fractions were submitted in triplicate to SDS-PAGE followed by electroblotting. Membranes were then probed with the specific antibodies. The immunoreactive material was developed with ECL ADVANCE® detection system, detected with a Bio-Rad Chemi Doc XRS apparatus, and quantified using the Quantity One 4.6.1 software (Bio-Rad Laboratories). The values of the arithmetical mean of the three determinations were considered only if the amounts were on a straight line.

3. Results

We have previously observed that calpain catalyzes the conservative proteolysis of native CFTR [14] and we have also established that in cells expressing F508del-CFTR the split channel form is accumulated in higher amounts as compared to controls [13]. To better characterize such proteolytic events we have analyzed the level of calpain activity in PBMC collected from controls and CF patients. These cells were selected due to the fact that F508del-CFTR is almost absent from plasma membrane, as observed in airway cells of CF patients, and that a defective immuno-response could be directly related to the severity of CF pathology [29]. PBMC were loaded with a calpain fluorogenic substrate and fluorescence intensity was continuously monitored for 20 min. In control cells (Fig. 1A), no significant changes in fluorescence were

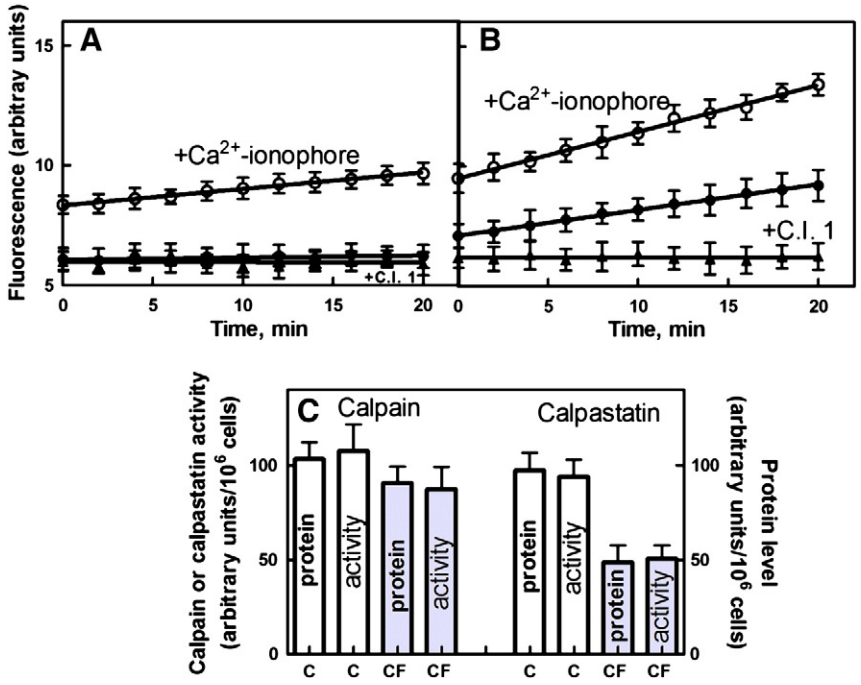

Fig. 1. Calpain and calpastatin system in PBMC from control and CF patients. Aliquots corresponding to 2×10^5 PBMC from 3 control (A) and 3 CF patients (B) were treated (as described in Section 2.3) to measure intracellular calpain activity in the absence (filled circles) or presence of 2 μM Ca^{2+}-ionophore (unfilled circles) or of 2 μM Ca^{2+}-ionophore and 1 μM C.I.1 (filled triangles). The values reported are the means ± S.D. for three different experiments. (C) Calpastatin activity was measured as reported in Section 2.4. Calpain activity has been assayed, following chromatographic separation of the protease from calpastatin, as previously described [19]. Aliquots corresponding to 2×10^5 PBMC from control (C) and CF patients (CF) were solubilized in Laemmli SDS/PAGE loading solution and submitted to 10% SDS/PAGE followed by immunoblotting. Calpain and calpastatin were detected using the specific antibodies and the protein bands were quantified as described in Section 2.7. The values of protein and activity quantification are reported as the means ± S.D. for three different experiments.

observed during the time of incubation. Calpain activity became detectable following exposure of C-PBMC to 2 μM Ca^{2+}-ionophore A23187. On the contrary, in cells from CF patients (Fig. 1B), the initial fluorescence intensity was higher than in controls and it progressively increased during the time of incubation. Following loading CF-PBMC with calcium in the presence of 2 μM Ca^{2+}-ionophore A23187 a further increase in the hydrolysis of the fluorogenic substrate was detected. In CF cells, preloaded with the synthetic calpain inhibitor-1 (C.I.1), the higher basal fluorescence and its progressive increase during the incubation time were both abolished, indicating that the two events are due to a calpain-mediated hydrolysis of the fluorogenic substrate. Comparing the rate of the fluorogenic substrate hydrolysis in untreated or Ca^{2+}-enriched control and CF-PBMC, it can be calculated that in CF cells calpain activity is at least 3–4 fold higher. Moreover, while in C-PBMC the basal calpain activity reached values corresponding to approximately 5% of the activity elicited by the Ca^{2+}-ionophore treatment, in untreated CF-PBMC it is close to half of the value measured in the presence of Ca^{2+}-ionophore. These observations support the hypothesis that, in cells carrying Phe508 deleted CFTR, Ca^{2+} homeostasis is sufficiently altered to promote a constitutive activation of calpain. This is consistent with the observation that calpain-mediated CFTR digestion is highly accelerated in CF-PBMC [13]. The increase in calpain activity observed in CF-PBMC exposed to Ca^{2+}-ionophore could be due to an overexpression of the protease or to a consumption of the natural inhibitor of calpain. Thus, we have measured both protein and activity of the two components of the proteolytic system. As shown in Fig. 1C, calpain activity was poorly affected in CF cells, whereas calpastatin was reduced to approximately 50% of controls, both as protein and inhibitory activity. This observation indicates an unbalance in the regulation of the Ca^{2+}-dependent proteolysis in CF-PBMC and is in agreement with the abnormal activation of calpain detectable in these cells.

To establish if this alteration in Ca^{2+} homeostasis and calpain activity in CF-PBMC could affect other members of the functional complex taking origin from CFTR at membranes [30,31], we have selected NHERF-1 scaffolding protein, since it represents the molecular bridge from proteins at the plasma membrane and other partners facing the cytoplasm compartment. As shown in Fig. 2A, in PBMC from healthy donors NHERF-1 was preferentially localized at the cell periphery. In particular, by scanning the green fluorescence of the confocal microscope images, it was established that approximately half of the total NHERF-1 was present at the cell periphery probably associated to plasma membranes (Fig. 2C). On the contrary, in PBMC from patients homozygous for F508del-CFTR, more than 95% of total NHERF-1 was distributed into the cytoplasm. It is remarkable that in CF-PBMC the alteration in NHERF-1 localization was qualitatively and quantitatively similar to that of F508del-CFTR (Fig. 2B and D). In fact, while in control cells approximately 40% of total CFTR was associated to plasma membranes and the remaining 60% was detected in endosome membranes [14], in cells from CF patients less than 5% of this protein was detectable on the plasma membranes, being almost all of the protein confined in a cytoplasmic localization. Thus, similar changes in cell distribution occurring to both proteins suggested a close interaction between CFTR and NHERF-1. Moreover, the absence of F508del-CFTR at the plasma membranes further supported the activation of calpain in CF-PBMC. To confirm this assumption we have analyzed the molecular forms of both CFTR and NHERF-1 in control and CF-PBMC.

As shown in Fig. 2E in C-PBMC, CFTR was present in a native and in a digested form. Using a mAb directed against the C-terminal of the protein, in addition to the native 170 kD, a larger amount of the 100 kD form was also detected as already reported in [14]. When we used a different antibody directed against the N-terminal region, a second 70 kD band was observed, indicating that CFTR is cleaved by calpain in two discrete fragments one of a 100 kD and the other of 70 kD, the latter corresponding to the N-terminal portion of the channel. Quantification of the CFTR bands revealed that the mean value of the ratios between the digested and native protein channel forms was 4.6 when we used the C-terminal antibody and 4.2 when we used the N-terminal antibody. Thus, the similarities in these values indicate that calpain promotes the cleavage of one or few

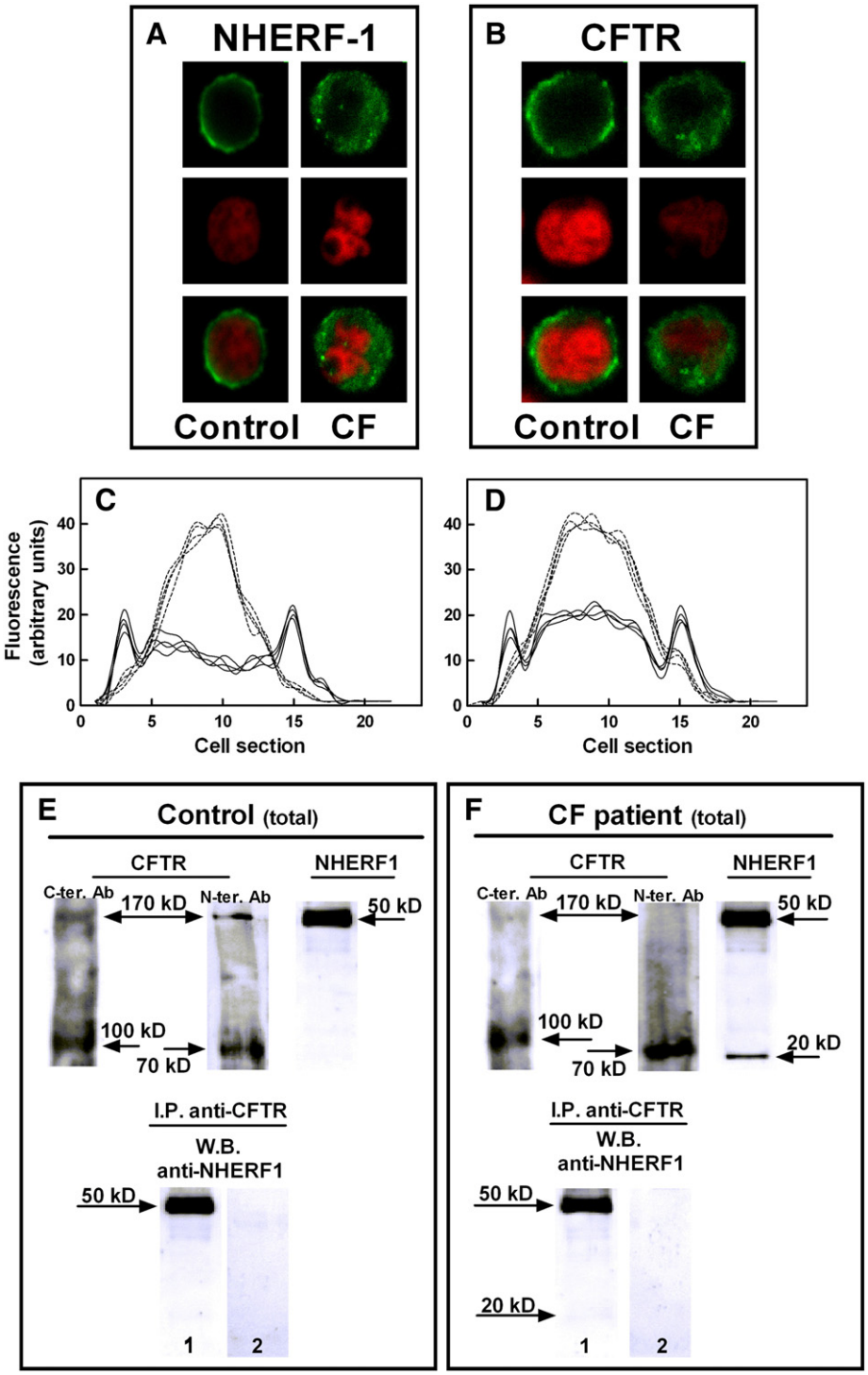

Fig. 2. Localization and association of NHERF-1 and CFTR in PBMC from control and CF patients. PBMC (10×10^6) from control and CF patients were fixed and permeabilized following the Triton/paraformaldehyde method [19]. (A) NHERF-1 localization was determined by confocal microscopy using anti-NHERF-1 antibody. (B) CFTR localization was determined by confocal microscopy using M3A7 mAb (see Section 2.5). Representative images are shown in both panels. The green fluorescence refers to the Ab stained protein and the red fluorescence refers to propidium iodide stained DNA (see Section 2.5). Fluorescence intensity was measured and quantified by cell scanning with Laser Pix Software as in [25]. Spectra of four cells from control (straight line) and from CF patients (dashed line) are reported (C and D). (E) Aliquots (20 μg) of the total material (see Section 2.6), prepared from PBMC of control or CF patients were solubilized in Laemmli SDS-PAGE loading solution, incubated at 37 °C for 30 min, and submitted to 6% SDS-PAGE followed by immunoblotting. CFTR forms were detected using M3A7 mAb (C-ter. Ab) or anti-CFTR polyclonal antibody (N-ter. Ab). (F) Alternatively, aliquots (20 μg) of the total material from control or CF-PBMC solubilized in Laemmli loading solution were heated at 95 °C for 5 min and submitted to 12% SDS-PAGE followed by immunoblotting. The different NHERF-1 forms were detected using anti-NHERF-1 antibody. The blot images shown are representative of four different experiments. (Lower panels) Total membrane fractions isolated from 10×10^6 control or CF PBMC were solubilized in 1% Triton® X-100 and immunoprecipitation was carried out using anti-CFTR mAb M3A7 or, as control, using only protein G-Sepharose (see Section 2.7). NHERF-1 from immunoprecipitation carried out with anti-CFTR mAb M3A7 (lanes 1) and NHERF-1 from immunoprecipitation with only protein G-Sepharose (lanes 2) were detected as in (E) and in (F).

peptide bonds located in between the first nucleotide binding domain and the regulatory domain of CFTR. These data are in agreement with previous observations indicating that calpain-generated CFTR fragments remained associated each other into the membrane moiety [14]. Similar analyses carried out on PBMC from patients homozygous for F508del-CFTR (Fig. 2F) showed that native 170 kD CFTR was

almost undetectable, although the split form containing both 100 kD and 70 kD fragments was still detectable in internal membranes.

Instead, NHERF-1 was detected in C-PBMC as a single form showing a mass of 50 kD, whereas in CF-PBMC the anti NHERF-1 antibody revealed, in addition to the native 50 kD form, the presence of a second protein band showing a mass of 20 kD. The interaction of NHERF-1 with native or split CFTR was explored by immunoprecipitation experiments (Fig. 2E and F, lower panels). Using an anti-CFTR antibody, it has been established that NHERF-1 was associated to CFTR both in control and CF patients. Since NHERF-1 was associated to CFTR also in CF-PBMC, in which the channel was present almost exclusively in the split form, it can be postulated that CFTR/NHERF-1 interaction occurs regardless of mutation as well as digestion and localization of the protein.

Moreover the same experiments revealed also that both native and 20 kD NHERF-1 forms were associated to the mutated CFTR split form, thus providing an explanation for the presence of NHERF-1 observed in cytoplasm of CF-PBMC and its absence in plasma membranes, as indicated by the intense cytosolic fluorescence. The low amount of 20 kD NHERF-1 detected in the immunoprecipitation experiments and shown in Fig. 2F could be due to a competition for binding to the immobilized CFTR between the two NHERF-1 forms, present in different amounts.

3.1. NHERF-1 is an "in vitro" calpain substrate

To demonstrate that the appearance of the 20 kD NHERF-1 form could be the result of a limited proteolytic attack occurring on the native form, we have investigated the involvement of calpain by exposing total membrane fractions from C-PBMC containing NHERF-1 to purified calpain. As shown in Fig. 3A, the native 50 kD protein band was 60–70% reduced and replaced by two additional bands having masses of 35 kD and 20 kD although showing different intensities. None of these digestion fragments were produced in the presence of C.I.1. A time course analysis (Fig. 3B) revealed that the rate of accumulation of the two proteins was different. In fact, the level of 20 kD band was, following 20 min of incubation, at least five-six fold higher than that of the 35 kD form. The formation of two different products could be the result of a two steps proteolytic process converting first the 50 kD form in the 35 kD fragment and then in the 20 kD form. Alternatively, the two products could be produced independently by a concomitant double attack of calpain. The two cleavage sites could be recognized by calpain with a different affinity, which could also explain their different accumulation. The hypothesis of the two cleavage sites was based on the fact that NHERF-1 can assume two distinct conformations depending on the context in which the protein is localized. In fact, it has been reported [31] that NHERF-1 could acquire a tight conformation when the two PDZ-binding domains and the ERM-binding domain are free and can be converted in a more relaxed form following association of ligands to such sites (Fig. 3C). On the basis of the position of the antibody epitope on the protein molecule, it can be established that calpain cleavage occurs between the two PDZ domains, producing the 35 kD fragment, or between the second PDZ domain and the ezrin binding domain, generating the 20 kD fragment (Fig. 3C). To verify whether NHERF-1 conformation was involved in addressing calpain on one or on the other cleavage site, NHERF-1 was removed from its association with CFTR complexes by washing total membrane fraction with 0.5 M NaCl in order to separate particulate from solubilized fractions. As shown in Fig. 3D, following incubation with purified calpain, the solubilized NHERF-1 was exclusively converted into the 20 kD fragment,

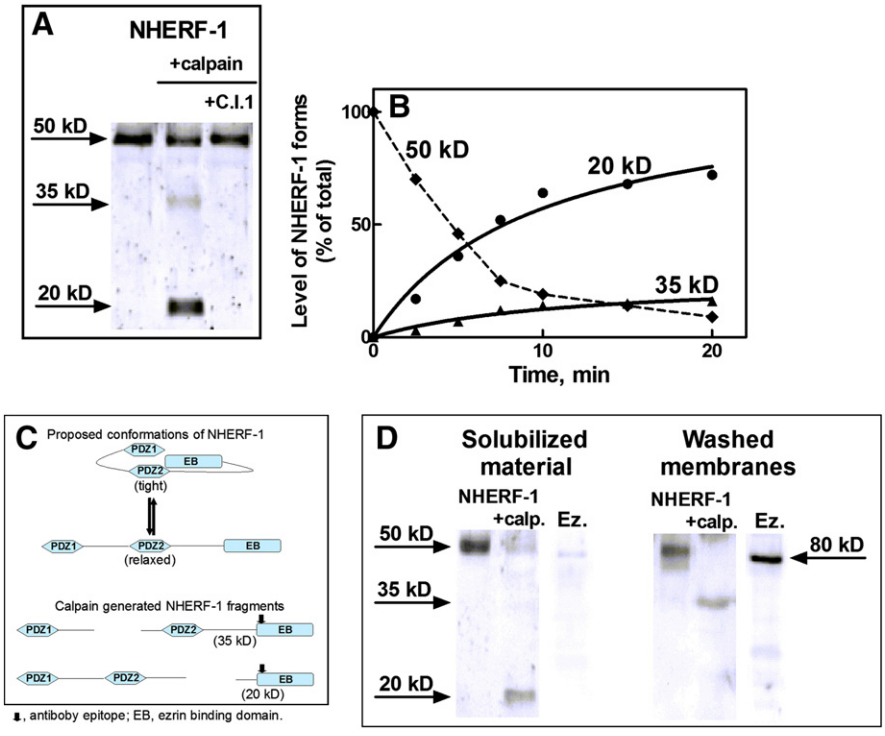

Fig. 3. Effect of ezrin on NHERF-1 digestion by calpain in CFTR containing complex. Aliquots (20 μg) of total membrane fractions (A), prepared from control PBMC as described in Section 2.6, were incubated at 37 °C for 5 min in the presence of 1 unit of isolated human erythrocyte calpain [16] and 1 mM $CaCl_2$. The reaction was stopped by solubilization and heating in Laemmli SDS-PAGE loading solution and the samples were submitted to 12% SDS-PAGE followed by immunoblotting. Ezrin and NHERF-1 forms were detected using the specific antibodies. The blot images shown are representative of three different experiments. (B) Aliquots (20 μg) of total membrane fraction prepared from C-PBMC were incubated as in (A) in the presence of human erythrocyte calpain for 5, 10 and 20 min. NHERF-1 forms were detected using the specific antibodies and quantified as described in Section 2.7. (C) Schematic representation of the conformational changes occurring on NHERF-1 [31] and the molecular organization of the NHERF-1 fragments generated by calpain. (D) Aliquots (20 μg) of total membrane fractions prepared from C-PBMC (D) were washed with 0.5 M NaCl as described in Section 2.6 and following centrifugation the supernatant (solubilized material) and the pellet (washed membranes) were separately collected and treated with calpain (+ calp.) as in (A).

whereas the membrane-associated scaffolding protein was converted into the 35 kD protein species, confirming the presence in NHERF-1 of two alternative calpain cleavage sites. The formation of the 35 kD fragment was observed only when ezrin was present in high amounts in the incubation mixture suggesting that the conformational change induced by association of NHERF-1 to ezrin [32], promoting an increase in the binding capacity of PDZ-domains, was directly related to the accessibility of calpain to the 35 kD generating cleavage site. Conversely, in preparations lacking ezrin, calpain preferentially produced the 20 kD NHERF-1 form (Fig. 3D).

3.2. Degradation of NHERF-1 by calpain in C-PBMC

To demonstrate if these NHERF-1 products were produced in cells following calpain activation, C-PBMC were loaded with Ca^{2+} by incubation with Ca^{2+}-ionophore A23187. As shown in Fig. 4A and B, in concomitance with the almost complete conversion of the 170 kD CFTR into the digested (100 kD + 70 kD) form, a 35 kD NHERF-1 protein band was accumulated. Digestion of both proteins was prevented by preloading C-PBMC with C.I.1 (data not shown). The high levels of ezrin present in these cells could explain the absence of the 20 kD (Fig. 4C). Thus, the association of NHERF-1 with its partner proteins addresses calpain only to the low affinity cleavage site generating the 35 kD fragment. These proteolytic events are accompanied by a redistribution of CFTR and NHERF-1, both becoming internalized into the cytoplasm (Fig. 4D and E). Altogether these findings are clearly indicating that CFTR functional cluster is target of calpain. However, the most sensitive substrate of the protease is CFTR and its limited proteolysis is required for the recycling of this protein in cytoplasm. NHERF-1 is also a calpain substrate but its fragmentation occur in conditions promoting a massive calpain activation as it occurs during exposure of cells to Ca^{2+}-ionophore or in CF-PBMC. Thus the accumulation of the 20 or the 35 kD fragments is indicative of a pathological condition mediated by calpain overactivation. However, the presence of the 20 kD fragment in CF cells is also indicating that the accessibility of calpain protein target in CFTR functional complex is also altered.

3.3. Correlation between the 20 kD NHERF-1 form and CF pathology

To establish if the presence of this scaffolding fragment is characteristic of cells containing the mutated F508del-CFTR, PBMC from 12 CF patients homozygous for F508del-CFTR mutation were analyzed together with PBMC from 10 healthy donors. As shown in Fig. 5A, native 50 kD NHERF-1 was present in cells from both control and CF patients. However, the mean values of NHERF-1 level in cells from CF patients was 1.1–1.2 fold higher than in controls, probably due to a higher expression of this scaffolding protein in CF-PBMC.

Instead, the 20 kD form was exclusively detected in cells from all CF patients and absent in cells from all control subjects. The amount of 20 kD NHERF-1 varied from 4 to 16% of total with an average value corresponding to approximately 10%. Altogether these observations indicate that the presence of the 20 kD NHERF-1 form could be considered typical for cells expressing the mutated F508del-CFTR. This conclusion is supported by the finding that in PBMC of heterozygous parents, the 20 kD NHERF-1 form is absent and the level of the native form is very similar to control (Fig. 5A and B). Since the formation of the 20 kD fragment seems to require the disorganization of the CFTR functional complex, we have analyzed the effect of Phe508 deletion in CFTR on the association of NHERF-1 to membranes. As shown in Fig. 6A, while in C-PBMC the 50 kD NHERF-1 form was equally distributed in both soluble and membrane fractions, in cells from CF patients the level of the soluble NHERF-1 protein was 1.5–1.6 fold higher than in controls whereas, the particulated form was five fold lower as compared to control cells ($P<0.0001$). The 20 kD NHERF-1 protein, present exclusively in CF-PBMC was detected in both cell fractions (Fig. 6B).

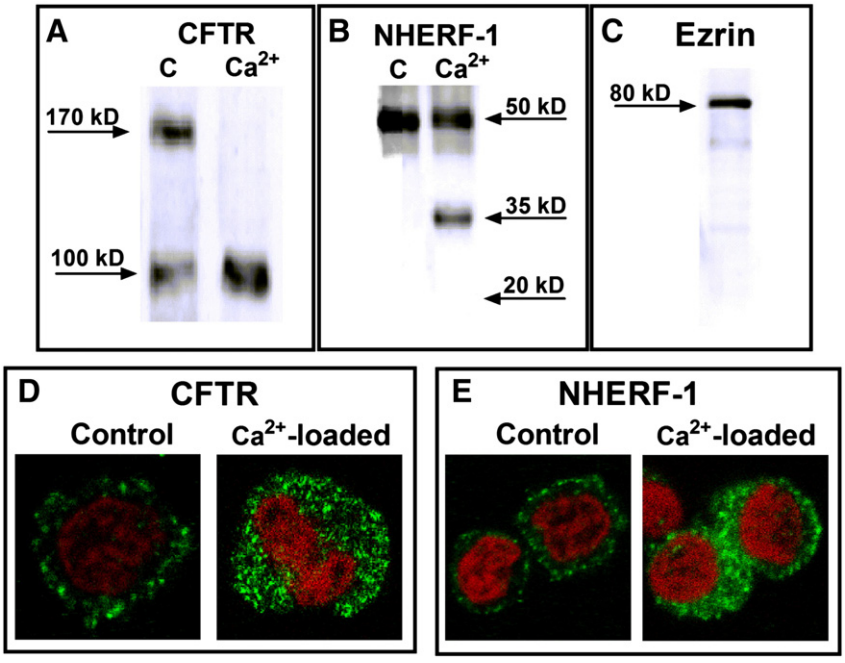

Fig. 4. Effect of NHERF-1 and CFTR digestion on their distribution in Ca^{2+}-loaded PBMC. (A) PBMC (10×10^6 cells) were treated with 2 μM Ca^{2+}-ionophore A23187 (Ca^{2+}-loaded) for 30 min at 37 °C in 10 mM Hepes pH 7.4, containing 0.14 M NaCl, 5 mM KCl, 5 mM Glucose and 2 mM $CaCl_2$. After treatment, aliquots (20 μg) of the incubation were solubilized in Laemmli SDS-PAGE loading solution, incubated at 37 °C for 30 min, and submitted to 6% SDS-PAGE followed by immunoblotting. CFTR forms were detected using M3A7 mAb. Alternatively, aliquots (20 μg) were solubilized in loading solution, heated at 95 °C for 5 min and submitted to 12% SDS-PAGE followed by immunoblotting. NHERF-1 (B) and ezrin (C) were detected using the specific antibodies. The blot images shown are representative of four different experiments. CFTR and NHERF-1 localization (D and E) in control and Ca^{2+}-loaded PBMC was determined by confocal microscopy using the specific antibodies (see Section 2.5). The images are representative of four different experiments.

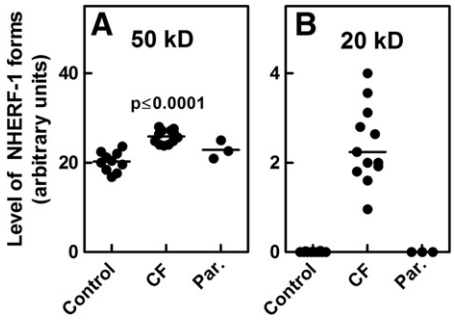

Fig. 5. Levels of 50 kD and 20 kD NHERF-1 in PBMC from control and CF patients. Aliquots (30 μg) of the total material (see Section 2.6), prepared from PBMC of 10 controls (control) or 12 CF patients (CF) or 3 heterozygous parents (Par.) were solubilized in Laemmli loading solution, heated at 95 °C for 5 min and submitted to 12% SDS-PAGE followed by immunoblotting. NHERF-1 forms were detected using anti-NHERF-1 antibody. The immunoreactive bands, corresponding to molecular masses of 50 kD (A) and 20 kD (B), were quantified as described in Section 2.7. Statistical analysis with Anova test for a single factor revealed that the 95% confidence intervals of the three groups did not superimpose showing a p-value ≤ 0.0001.

These findings indicate that the Phe508 deletion induces a lower affinity of CFTR for NHERF-1 resulting in disassembly of the complex and explaining the higher susceptibility of NHERF-1 to calpain digestion. The data shown in Fig. 6C and D demonstrate this assumption because NHERF-1 can be washed out from membranes only if the mutated and split CFTR is accumulated.

Moreover, preliminary observations show that ezrin, a known calpain substrate [33], is significantly reduced in CF-PBMC (Fig. 7),

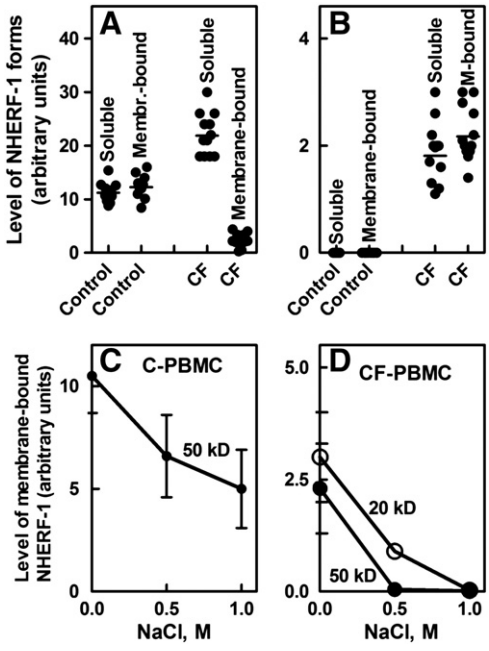

Fig. 6. Level and distribution of NHERF-1 forms in PBMC from control and CF patients. Aliquots (20 μg) of soluble and membrane fractions (see Section 2.6) prepared from PBMC of 10 controls (Control) and 12 CF patients (CF) were solubilized in Laemmli loading solution, heated at 95 °C for 5 min and submitted to 12% SDS-PAGE followed by immunoblotting. NHERF-1 forms were detected using anti-NHERF-1 antibody. The immunoreactive bands, corresponding to the molecular masses of 50 kD (A) and 20 kD (B), were quantified as described in Section 2.7. Aliquots (20 μg) of membrane and washed membrane fractions (see Section 2.6) prepared from PBMC of 4 controls (C) and 4 CF patients (CF) were solubilised, heated and submitted to 12% SDS-PAGE followed by immunoblotting. NHERF-1 forms were detected using anti-NHERF-1 antibody. The immunoreactive bands, corresponding to molecular masses of 50 kD and 20 kD, were quantified as described in Section 2.7 and the values are reported as the arithmetic mean ± S.D.

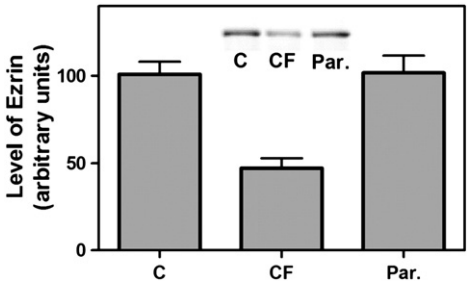

Fig. 7. Level of ezrin in PBMC from control and CF patients. Aliquots (20 μg) of total material prepared from PBMC of 4 controls (C), 4 CF patients (CF) and 3 heterozygous parents (Par.) were solubilised, heated and submitted to 12% SDS-PAGE followed by immunoblotting. Ezrin was detected using 18/Ezrin mAb and the immunoreactive bands were quantified as described in Section 2.7.

further demonstrate the disorganization of the CFTR complex as in this condition NHERF-1 assumes the conformation convertible by calpain into the 20 kD fragment.

4. Discussion

A number of transmembrane channel proteins display PDZ-binding modules recognized by scaffolding proteins and operating as linkers between the channels and other proteins generating functional complexes [6–9].

Thus, the scaffolding proteins assemble these complexes concentrating and positioning transporters and enzymes in close proximity to their substrates or regulatory proteins, avoiding non specific interactions and favouring signal transduction processes [1,2].

CFTR generates a specific functional protein complex through the association to NHERF-1 protein which then recruits in addition to other channels, cytoskeletal elements (ezrin, radixin, moesin) and PKA, the regulator of the CFTR channel activity [10]. The functional relationship between CFTR and NHERF-1 is further demonstrated by the increase of F508del-CFTR in plasma membrane following overexpression of this scaffolding protein [34,35].

It has been reported that many transmembrane ion channels, such as NMDAR, CFTR, AMPA, L-type Ca^{2+}-channels and other components of their complexes are substrate of calpain [11,12], which catalyzes a conservative proteolysis usually resulting in functional changes of the target protein [36]. We have observed that calpain could be inserted into these protein clusters through the chaperone HSP90 that associates with the protease reducing its sensitivity for Ca^{2+} ions [37]. Through this interaction the protease is brought in close proximity of its substrates [37]. Calpain digestion of these functional complexes could be involved in the control of the transmembrane ion conductance. This is supported by the fact that calpain-mediated limited proteolysis of CFTR occurs in growing cells as well as in circulating lymphocytes and following digestion the channel is recycled into the cytoplasm [14]. A similar process is also suggested to occur for NMDA receptor [12].

On the basis of these findings we are now proposing a new physiological role of calpain in controlling the level of ion channels at plasma membrane and thereby the ion flux across these structures. Accordingly, it can be postulated that an abnormal intracellular calpain activation could result in a massive increase in the degradation rate of a specific ion channel, promoting an almost complete removal of this protein from plasma membrane. In this condition calpain changes from a physiological to a pathological role. Experimental evidence in support of this calpain function has been obtained by analyzing PBMC of patients homozygous for the F508del-CFTR. We are here reporting that calpain is abnormally active in CF-PBMC due to an alteration in the protease regulatory mechanism probably

accompanied also by increase in $[Ca^{2+}]_i$[38]. In these conditions proteolysis and internalization of the mutated CFTR are highly accelerated causing the absence of the channel from its functional localization at the plasma membrane. We have also established for the first time that NHERF-1, a CFTR partner, is a substrate of calpain that recognizes two sites differing for their affinity for the protease. The accessibility of these cleavage sites is dependent on the NHERF-1 conformation which is affected by protein interaction at the PDZ and ERM domains. Thus calpain can convert NHERF-1 into different products showing masses of 20 and 35 kD. It is relevant the fact that in normal cells following calpain activation NHERF-1 digestion generates exclusively the 35 kD digested form, whereas in CF cells, in which calpain is already active, only the 20 kD form is produced. Although the presence of one or the other NHERF-1 fragment is indicative of an overactivation of calpain and of its pathological function, it remains to establish why the 35 kD fragment is never detectable in CF patients cells. Our observations are suggesting that this is the result of a reduced affinity of the mutated CFTR, regardless if it is split or not, for NHERF-1 causing a disorganization of the CFTR functional complex. In this altered organization of the cluster calpain recognizes, in addition to its natural substrate CFTR, also NHERF-1 generating the 20 kD form as well as ezrin. The susceptibility of these proteins to calpain proteolysis opens a new field of investigation on its possible involvement in cell transformation since it has been suggested that NHERF-1 can act also as a tumour suppressor [39–44].

Moreover, the pathological role of calpain in CF cells could be further increased by the effect of its activation in cell pulmonary infections. Indeed, a recent report indicates that calpain inhibition prevented staphylococcal penetration through monolayers of airway epithelial cells [45].

The transition from a physiological to a pathological calpain function can occur also in nervous cells mediated by the digestion of the Ca^{2+}-channels NMDA or AMPA receptors [11,46].

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