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Calpain digestion and HSP90-based chaperone protection modulate the level of plasma membrane F508del-CFTR

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ABSTRACT

We are here showing that peripheral mononuclear blood cells (PBMC) from cystic fibrosis (CF) patients contain almost undetectable amounts of mature 170 kDa CF-transmembrane conductance regulator (CFTR) and a highly represented 100 kDa form. This CFTR protein, resembling the form produced by calpain digestion and present, although in lower amounts, also in normal PBMC, is localized in cytoplasmic internal vesicles. These observations are thus revealing that the calpain-mediated proteolysis is largely increased in cells from CF patients. To characterize the process leading to the accumulation of such split CFTR, FRT cells expressing the F508del-CFTR mutated channel protein and human leukaemic T cell line (JA3), expressing wild type CFTR were used. In in vitro experiments, the sensitivity of the mutated channel to the protease is identical to that of the wild type, whereas in Ca^{2+}-loaded cells F508del-CFTR is more susceptible to digestion. Inhibition of intracellular calpain activity prevents CFTR degradation and leads to a 10-fold increase in the level of F508del-CFTR at the plasma membrane, further indicating the involvement of calpain activity in the maintenance of very low levels of mature channel form. The higher sensitivity to calpain of the mutated 170 kDa CFTR results from a reduced affinity for HSP90 causing a lower degree of protection from calpain digestion. The recovery of HSP90 binding capacity in F508del-CFTR, following digestion, explains the large accumulation of the 100 kDa CFTR form in circulating PBMC from CF patients.

1. Introduction

It is well established that cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP regulated chloride channel located at the apical surface of the cell [1–3]. Its level and functional location is the result of a complex multistep process involving synthesis, maturation and correct folding of the protein [4,5], finally undergoing targeting at the apical plasma membrane [6–9].

Deletion of Phe^{508} in CFTR (F508del-CFTR) is the major cause of cystic fibrosis (CF) [10]. Due to an inefficient folding, a large number of the mutated protein molecules is destroyed by the ubiquitin proteasome system as part of the endoplasmic reticulum (ER)-associated quality control (ERAD) [11]. In addition, structurally destabilized membrane F508del-CFTR molecules that have escaped the ERAD can be removed by lysosomal degradation in the course of the plasma membrane endocytic recycling of CFTR [12–15]. As a result of the action of both ERAD and peripheral quality control, a very low amount of F508del-CFTR is functionally present at the plasma membrane and an excess of nonfunctional protein remains trapped within the cell [15]. Furthermore, using an in vitro biochemical assay, it has been observed that the cytoplasmic domain of both wild type and F508del-CFTR, expressed in rabbit reticulocyte lysates, undergoes proteolytic degradation by a still unidentified cysteine proteinase [16].

Since misfolding is the intrinsic molecular defect responsible for the instability of the mutated F508del-CFTR, several efforts were made to develop chemical compounds, usually defined as folding correctors, capable to restore a proper CFTR molecular folding thus being potentially useful as therapeutic devices in cystic fibrosis [17–21]. These compounds, although capable to deliver only small amounts of functional F508del-CFTR at the plasma membrane, resulted to be very useful in providing new information concerning the mechanisms involved in the maturation of CFTR. Since the conformational defect of F508del-CFTR was shown to be temperature-dependent, the processing block in ER could be, at least partially, overcome reducing the temperature at 27 °C [22]. The observation that, as a consequence of rescuing F508del-CFTR by low temperature and by correctors, a decrease in the altered intracellular $[Ca^{2+}]$ occurs [23], could suggest that an additional proteolytic system is involved in the regulation of CFTR trafficking.

Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated degradation; CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; C.I.2, calpain inhibitor 2

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We have recently presented data showing that, in growing cells, in addition to the native 170 kDa and to the immature 150 kDa CFTR forms, an additional one with a Mr of 100 kDa was also present. This 100 kDa CFTR form has never been identified before among the various CFTR forms [24]. However, similar 100 kDa CFTR fragment has been obtained by incubation of the membrane-bound channel protein with trypsin, indicating that CFTR exposes, in the inner surface of the plasma membrane, a sequence highly accessible to proteases [24,25]. The calpain-mediated proteolytic origin of the 100 kDa CFTR form has been supported by the increase in the native 170 kDa form, localized at the plasma membrane, observed in cells grown for two cell cycles in the presence of a synthetic inhibitor of the protease (C.I.2) or in cells over-expressing calpastatin, the natural protein inhibitor of calpain [24]. Based on the molecular mass of the fragments, produced by proteolysis, the calpain site of cleavage can be located between the first nucleotide binding domain (NBD1) and the regulatory domain. However, the fragments, present in the split protein, remain associated to membranes in close contact each other. Of relevance was the finding that, also in peripheral mononuclear blood cells (PBMC) from healthy donors, the 100 kDa CFTR form not only was present, but its level was higher than that of the native channel protein. Thus, degradation of plasma membrane CFTR appears to be normally operating in physiological conditions.

We are herewith showing that, in cells expressing F508del-CFTR, also the mutated channel protein undergoes an identical calpain-mediated limited proteolysis which occurs however at a much higher extent producing an even larger excess of the 100 kDa CFTR form. This calpain-mediated F508del-CFTR degradation can thus be considered involved in the maintenance of a very low level of the mutated chloride channel at the plasma membrane. In accordance, data are presented demonstrating that inhibition of calpain restores large amounts of F508del-CFTR at the plasma membrane.

We are suggesting that, beside ERAD and the peripheral quality control are responsible for the removal of misfolded channel molecules, degradation by calpain of the mutated F508del-CFTR, present at the plasma membrane, is an additional mechanism for the modulation of the channel protein level at its functional location. Accordingly, the Ca^{2+}-dependent proteolysis can be considered as a promising target for CF therapeutic treatment.

2. Materials and methods

2.1. Materials

Nutrient mixture F-12 Coon's modification, RPMI1640, leupeptin, calpain inhibitor 2 (C.I.2), calcium ionophore A23187, Triton® X-100, dibutyryl cAMP and MG132 were purchased from Sigma-Aldrich. Foetal bovine serum (FBS), penicillin, streptomycin and L-glutamine were obtained from EuroClone. Zeocin was purchased from Invivogen. Geneticin was obtained from Invitrogen. 4-(2-aminoethyl) benzene-sulfonylfluoride (AEBSF) was obtained from Calbiochem. ECL ADVANCE® Detection System was obtained from GE Healthcare. t-Boc-Leu-Met-CMAC fluorogenic calpain substrate was purchased from Molecular Probes (Invitrogen). Corr-4a was kindly provided Dr. L.J. Galietta, Molecular Genetics Laboratory, Giannina Gaslini Institute Genova, Italy. Human erythrocyte calpain was isolated and assayed as reported in [26]. One unit of calpain activity is defined as the amount that releases 1 nmol/h of free α-amino groups under the specified conditions [27]. HSP90 was purified from rat brain as reported in [28].

Anti-CFTR monoclonal antibody (clone M3A7) was purchased from Millipore. Anti-HSP90 monoclonal antibody was obtained from BD Biosciences.

2.2. Cell culture

Fisher rat thyroid (FRT) cell line expressing human wild type (WT) or F508del-CFTR was cultured at 37 °C (5% CO_2) with Coon's F-12 growth medium, containing 10% FBS, 10 U/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine and 0.6 mg/ml zeocin. FRT cell line was kindly provided by Dr. L.J. Galietta, Molecular Genetics Laboratory, Giannina Gaslini Institute Genova, Italy. JA3 cells, a human leukaemic T cell line, were cultured at 37 °C (5% CO_2) with RPMI1640 growth medium, containing 10% FBS, 10 U/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine.

2.3. Isolation of the total membrane fraction

FRT cells expressing WT or F508del-CFTR, JA3 cells (collected during the growing log-phase) and PBMC isolated as previously reported [29], were washed three times with ice-cold PBS solution.

The total membrane fraction was isolated following lysis of 25×10^6 cells in 1 ml buffer A (1 mM EDTA, pH 7.0), containing 5 μg leupeptin and 10 μg AEBSF by three cycles of freezing and thawing, followed by centrifugation at $60\,000\times g$ for 10 min at 4 °C. The particulate material (total membrane fraction) was washed three times in buffer A and finally suspended in 0.5 ml of the same solution. The protein concentration was determined following the Lowry procedure, and aliquots (50 μg protein) were diluted in 30 μl Laemmli SDS-PAGE loading solution, incubated at 37 °C for 30 min and submitted to 6% SDS-PAGE [30].

2.4. Native electrophoresis

The total membrane fraction, prepared as described above, from JA3 cells and PBMC from control donors and CF patients (F508del-CFTR homozygotes), was solubilized in 100 μl 0.35 M Tris/HCl pH 9.0, containing 0.5 M KCl and 0.1% Triton® X-100. The samples were then loaded on to a 0.8% agarose gel as described in ref. [24].

2.5. Isolation of plasma and intracellular membranes

The total membrane fraction (0.5 ml) isolated from JA3 cells and from FRT cells expressing WT or F508del-CFTR was fractionated on a discontinuous sucrose density gradient as described in [24].

2.6. Immunoprecipitation and immunoblotting

Plasma membranes, isolated from JA3 cells and from FRT cells expressing F508del-CFTR both collected during the growing log-phase, were solubilized in 300 μl buffer A containing 0.15 M NaCl and 1% Triton® X-100. Aliquots of HSP90 purified from rat brain [28] were then added to the membrane suspensions. The mixtures, pre-treated with protein G-Sepharose, were incubated over night with 2 μg of anti-CFTR antibody (clone M3A7) at 4 °C. Protein G-Sepharose was then added to the samples which were incubated for an additional 1 h with continuous gentle shaking. The immunocomplexes were washed three times with buffer A, incubated at 37 °C in SDS-PAGE loading buffer for 30 min and submitted to SDS-PAGE (6% gels) [30]. Proteins were then transferred to nitrocellulose membranes (Bio-Rad) by electroblotting [31] and probed with the specific mAb, followed by a peroxidase-conjugated secondary antibody as described in [32]. 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).

2.7. Confocal microscopy imaging and fluorescence quantification

Cells (10^5) were fixed and permeabilized by the Triton/paraformaldehyde method, as described in ref. [33]. Cells were treated with 10 μg/ml CFTR antibody (M3A7) 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. Images were collected using a Bio-Rad MRC1024

confocal microscopy, with a 60× Plan Apo objective with numerical aperture 1.4. 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 ref. [34].

2.8. Assay of intracellular calpain activity

FRT cells expressing WT or F508del-CFTR (2×10^4 cells) grown on a 96-well microplate or, alternatively, JA3 cells (10^5) were incubated for 30 min at 37 °C in 100 µl buffer B (10 mM Hepes pH 7.4, 0.14 M NaCl, 5 mM KCl, 5 mM glucose) containing 50 µM t-Boc-Leu-Met-CMAC, the fluorogenic substrate, in the absence or presence of 20 µM dibutyryl cAMP, known to favour the protease activation by removal of calpastatin from cytosol [35,36]. Cells were then washed twice with buffer B in order to remove substrate excess and after addition of 100 µl buffer B containing 2 mM $CaCl_2$ and, alternatively, 0.1 or 0.5 µM calcium ionophore A23187. Calpain activity was assayed after 10 min of incubation and expressed as the increase in fluorescence detected at 37 °C with a Mithras LB 940 plate reader (Berthold Technologies). The excitation/emission wavelengths were 355/485 nm, respectively.

3. Results

3.1. Characterization of the CFTR forms in growing cells expressing the wild type and Phe^{508} deleted species

In peripheral mononuclear blood cells (PBMC) from healthy subjects three major CFTR bands have been previously identified [24]. A first one, corresponding to the native mature 170 kDa form (band C), a second one of 150 kDa, generally indicated as an immature channel form (band B) and a third one of a molecular mass of 100 kDa, characterized as a fragment produced by a calpain-mediated limited proteolysis [24]. At difference, in PBMC from cystic fibrosis (CF) patients, the 170 kDa and the 150 kDa forms were represented by two very faint bands, whereas the 100 kDa CFTR fragment was the most largely detectable form exceeding in amount 3-fold to 4-fold the level present in control PBMC (Fig. 1A and B). These findings are indicating that, in PBMC of CF patients, the mutated F508del-CFTR undergoes to calpain-mediated digestion at a much higher extent in comparison with controls, thereby confirming the constitutive occurrence of this degradative process. To further explore the role of calpain digestion on the functional level of F508del-CFTR and to overcome the experimental difficulties derived by the use of nongrowing cells and particularly by the very low level of detectable mutated channel form, we have utilized FRT cells expressing both wild type or F508del-CFTR and a human leukaemic T cell line (JA3 cells) constitutively expressing the wild type CFTR. These clones were selected because in each one the native 170 kDa or the split 100 kDa forms were either both present or one of the two was highly predominant [24], thereby allowing more precise comparative studies. In Fig. 1 the immunoblot analyses performed on total membrane fraction, isolated from the three cell types, are also reported. In FRT cells, probably due to a large expression of the wild type CFTR, most of the channel protein was present in the 170 kDa form and only low amounts of the 150 kDa CFTR and 100 kDa forms were detectable (Fig. 1C). In the same cell

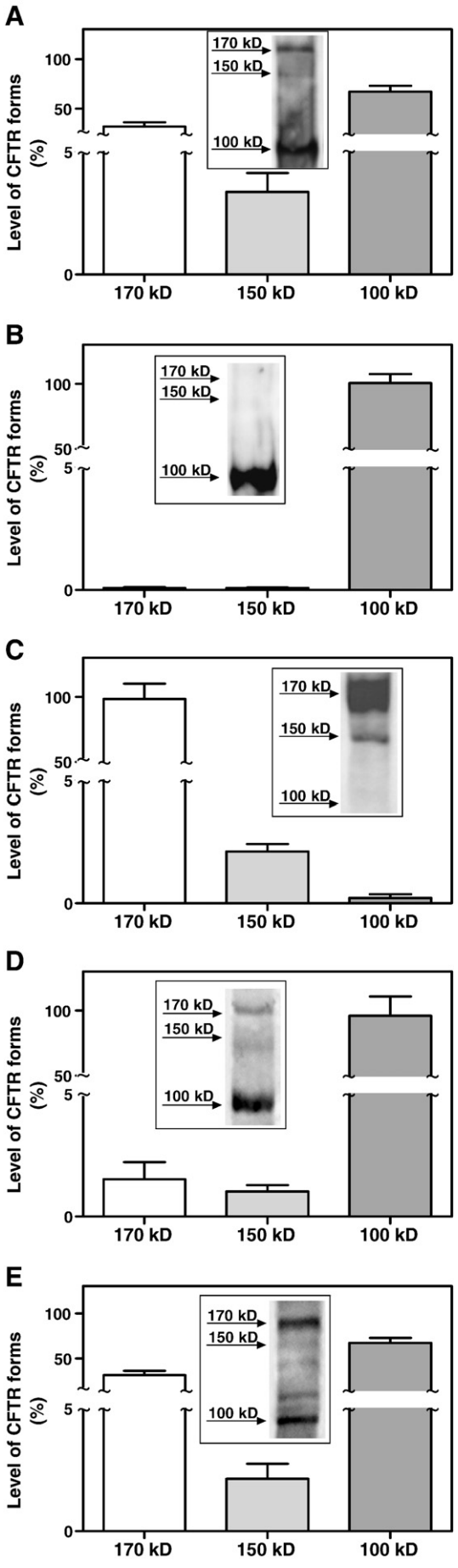

Fig. 1. CFTR forms in PBMC, FRT and JA3 cells. Aliquots (20 µg) of the total membrane fraction (see Materials and methods), prepared from normal (A) or CF (B) PBMC, from FRT cells expressing WT (C) or F508del-CFTR (D) and JA3 cells (E), were solubilized in Laemmli SDS-PAGE loading solution, incubated at 37 °C for 30 min and submitted to 6% SDS-PAGE followed by immunoblotting. The arrows indicate the 170, 150 and 100 kDa CFTR forms detected using M3A7 mAb and the immunoreactive bands were quantified as described in Materials and methods. The values are reported as the arithmetic mean ± SD. of three different experiments. The images are representative of three different experiments.

line expressing the F508del-CFTR, more than 90% of the channel corresponded to the 100 kDa species, whereas the 170 kDa and 150 kDa forms were represented in very low amounts (Fig. 1D). Finally, as shown in Fig. 1E, in JA3 cells both native and split CFTR were present, each one in an amount similar to that observed in circulating normal PBMC, indicating that the 100 kDa form is produced in physiological conditions.

3.2. Cellular localization of different CFTR forms

In Fig. 2A are reported the confocal microscope images establishing the cellular localization of the different CFTR forms present in all the cell types examined. In PBMC from healthy subjects (containing both 170 kDa and 100 kDa CFTR forms) the CFTR-dependent fluorescence was present at the plasma membrane as well as throughout

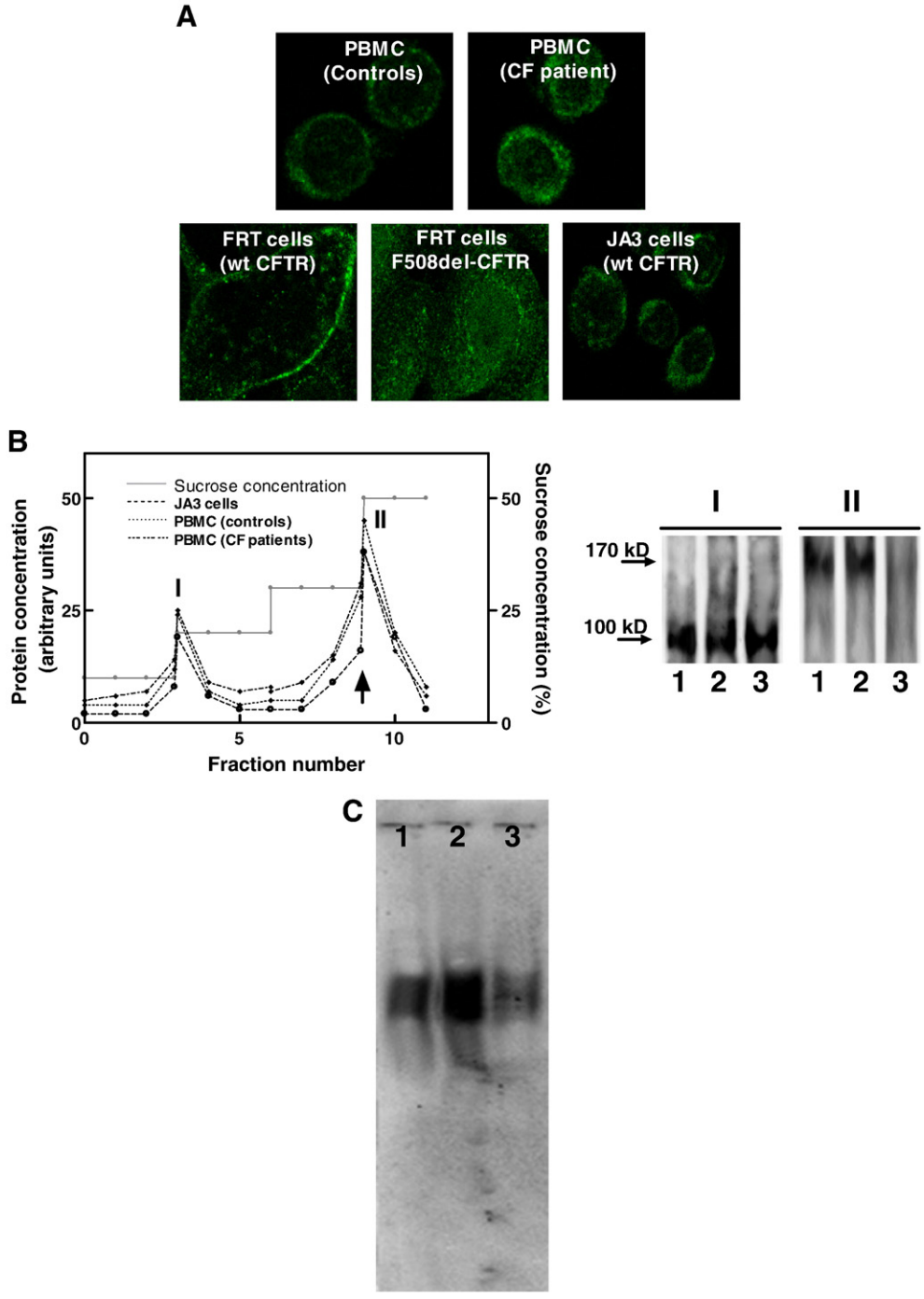

Fig. 2. Intracellular localization of different CFTR forms. (A) CFTR localization was determined in PBMC, in FRT cells expressing F508del and WT CFTR and in JA3 cells, by confocal microscopy using M3A7 mAb (see Materials and methods). The images are representative of six different experiments. (B) Total membrane fraction (0.5 ml) prepared from JA3 cells (1) or PBMC from control subjects (2) or CF patients (3), as described in Materials and methods, was fractionated on a discontinuous sucrose density gradient (see Materials and methods). Membrane fractions were separately collected and the protein concentration was determined following the Lowry procedure. Peak I contains intracellular membranes and peak II contains plasma membranes: 5′-nucleotidase (5′-NT) activity was assayed [51]. Aliquots (30 μl) of each sample of intracellular and plasma membranes 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 was detected using mAb M3A7. (C) Aliquots (20 μg) of the total membrane fraction prepared for native electrophoresis (see Materials and methods) from JA3 cells (lane 1) and PBMC from control subjects (lane 2) and CF patients (lane 3) were loaded on to a 0.8% agarose gel followed by immunoblotting as described in Materials and methods. CFTR was detected using the specific antibody (clone M3A7).

the cell cytoplasm. In PBMC from CF patients (containing almost exclusively the 100 kDa CFTR form) the channel protein was almost exclusively diffused into the cytoplasmic space. These findings indicate that the mature 170 kDa CFTR form is localized at the plasma membrane and that the fluorescence signals of the digested form become detectable in a punctate pattern through the cytoplasm, suggesting an association with the endomembrane system. This conclusion received support by the confocal images of CFTR expressed in FRT and in JA3 cells. In fact, in FRT cells expressing the wild type channel form of 170 kDa, the protein was confined almost exclusively at the plasma membrane level, whereas in FRT cells expressing the F508del-CFTR (in which the 100 kDa CFTR was the predominant form) the fluorescence, accordingly to previous observations [37], appeared to be diffused throughout the cell cytoplasm. Moreover, in JA3 cells (in which the native and the digested 100 kDa CFTR forms are both present) the protein channel was found to be present both at the plasma membrane and within the cytoplasm. In addition to confocal microscope analysis, the localization of the different CFTR forms was further explored in isolated cell membranes. To this purpose, total membrane fraction isolated from PBMC of normal subjects and of CF patients was submitted to a sucrose density gradient centrifugation previously set [24] to separate plasma membranes from low density internal ER membranes. JA3 cell membranes were used as control [24]. As shown in Fig. 2B, the 170 kDa CFTR forms were exclusively present in the high density fraction corresponding to plasma membrane, whereas the 100 kDa CFTR species were detected in the low density membrane fraction. Altogether these data are thus consistent with previous observations indicating that the 100 kDa form produced by calpain digestion was recovered in the internal membrane vesicles [24]. Furthermore, since our observations suggested that the 100 kDa CFTR species were the product of an intracellular limited proteolysis exerted by calpain, we have explored if this protein was a discrete form. We have found (Fig. 2C) that the charge–mass ratio of CFTR, evaluated on total membrane fraction by electrophoresis in non-denaturating conditions, was not modified by the presence of large amounts of the 100 kDa species. This finding indicates that the proteolytic split, occurring in CFTR molecule, produces a very limited number of products which remains associated each other into the membrane moiety.

3.3. Susceptibility of F508del-CFTR to calpain digestion

All evidences so far presented indicated that both native and mutated CFTR were digested by calpain and converted in a split form identifiable as a 100 kDa protein. It remained to be established if the Phe^{508} deletion altered the sensitivity of the chloride channel to calpain. To this purpose, isolated total membrane fractions from both FRT cells expressing wild type or F508del-CFTR forms and from JA3 cells were exposed to purified calpain and the changes in the molecular properties of CFTR forms were evaluated in western blot analysis. The data reported in Fig. 3 indicated that wild type 170 kDa CFTR was totally converted and only partially recovered as the 100 kDa form. This finding can be explained by the observation that, when this fragment was pre-existing in cell membranes, it was degraded by the protease at lower rate (see JA3 cells). The digestion pattern of F508del-CFTR resulted to be almost identical to that of the wild type, suggesting that the deletion did not induce the formation of different fragments. Addition of a synthetic calpain inhibitor prevented proteolysis of all CFTR forms (data not shown), thus excluding the involvement of contaminating plasma membrane proteases. A comparative analysis of the in vitro digestion kinetics (Fig. 3B) indicated that the rate of degradation of the F508del-CFTR was very similar to that of the wild type form and the 100 kDa fragment, generated from both CFTR forms, was more resistant to

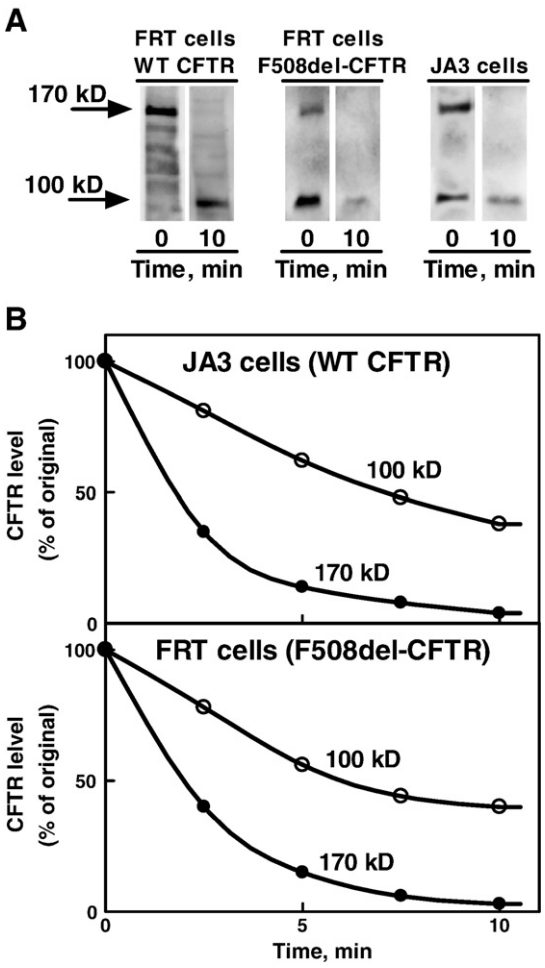

Fig. 3. In vitro digestion of CFTR from FRT and JA3 cells by calpain. (A) The total membrane fraction from FRT cells expressing wild type (5 μg) and F508del-CFTR (20 μg) and from JA3 cells (20 μg) was incubated at 37 °C for 10 min in the presence of 1 unit of calpain and then submitted to 6% SDS-PAGE followed by immunoblotting. CFTR was detected using mAb M3A7. (B) The total membrane fractions (20 μg) from JA3 cells containing WT CFTR and from FRT cells expressing F508del-CFTR were incubated in the conditions described in (A) for the indicated times. The samples were subjected to immunoblot analysis and CFTR was detected using mAb M3A7. The immunoreactive bands, corresponding to Mr of 100 kDa and 170 kDa, were quantified as described in Materials and methods.

further degradation. The similar sensitivity to calpain digestion could be explained by the fact that the cleavage site is not affected by the Phe^{508} deletion.

In FRT cells expressing F508del-CFTR, Ca^{2+} loading (Fig. 4A) induced a proteolytic digestion pattern identical to that observed in in vitro conditions using isolated membranes exposed to purified calpain. Intracellular involvement of calpain was further demonstrated by preloading cells with a permeable synthetic calpain inhibitor (C.I.2) in which the proteolysis resulted to be completely prevented (Fig. 4A). Identical effect was obtained using different calpain inhibitors such as calpain inhibitor-1 (C.I.1), PD151746 and E-64 (data not shown). In spite of a similar sensitivity to calpain, when the rate of degradation of the wild type and of the F508del-CFTR forms was comparatively analyzed in intact Ca^{2+}-loaded cells, it was observed (Fig. 4B) that the 170 kDa F508del-CFTR was degraded more rapidly than the wild type form. The digestion of the 100 kDa fragment proceeded at a similar rate, being the two fragments molecularly identical (Fig. 4C). Differently from the others CFTR forms, the level of the immature 150 kDa species, was unaffected following calpain activation (Fig. 4A).

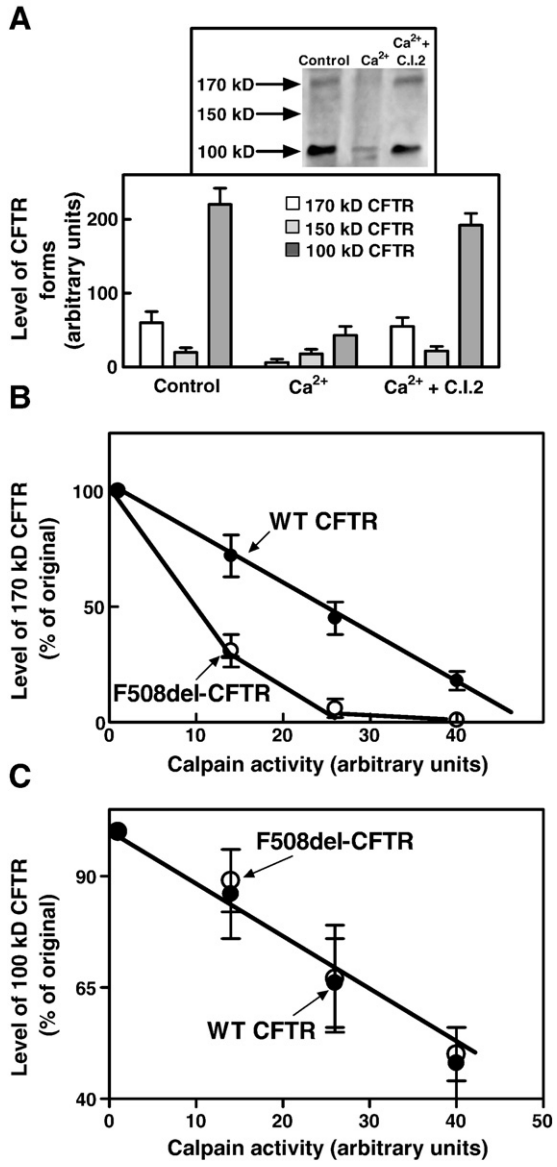

Fig. 4. Effect of calpain activation on CFTR levels in JA3 cells and in FRT cells expressing F508del-CFTR. (A) FRT cells (2×10^6) expressing F508del-CFTR were treated in culture medium (see Materials and methods) for 1 h at 37 °C with 1 μM Ca^{2+}-ionophore A23187 in the absence (Ca^{2+}) or the presence of 1 μM C.I.2 (Ca^{2+} + C.I.2). Aliquots (40 μl) of the total membrane fraction were solubilized in Laemmli SDS-PAGE loading solution, incubated at 37 °C for 30 min and then submitted to 6% SDS-PAGE, followed by immunoblotting. CFTR was detected using mAb M3A7 and the immunoreactive bands were quantified as described in Materials and methods. The values are reported as the arithmetic mean ± SD of three different experiments. The images are representative of three different experiments. (B and C) FRT cells (2×10^4) expressing F508del-CFTR or JA3 cells (10^5) were incubated for 30 min at 37 °C in buffer B containing 50 μM t-Boc-Leu-Met-CMAC fluorogenic substrate. Cells were then washed and suspended in 100 μl buffer B. Calpain activity was measured after 10 min of cells exposure to 2 mM $CaCl_2$ and 0.1 or 0.5 μM calcium ionophore A23187. Alternatively, the cells were pre-treated with 20 μM dibutyryl cAMP (see Materials and methods) and calpain activity was measured after 10 min of exposure to 2 mM $CaCl_2$ and 0.5 μM calcium ionophore A23187. In parallel, the cells were treated in culture medium in the same conditions used for assay intracellular calpain activity. Cells were then lysed and aliquots (20 μg) of the total membrane fraction was solubilized in Laemmli SDS-PAGE loading solution and submitted to SDS-PAGE 6%. Following immunoblotting, CFTR was detected with mAb M3A7 and the immunoreactive bands corresponding to Mr of 170 kDa and 100 kDa were quantified as described in Materials and methods. The values reported are the arithmetic mean ± SD of three different experiments.

3.4. Role of HSP90 in the regulation of CFTR

The findings so far presented have indicated that in vitro the sensitivity and the degradation pattern of the wild type and mutated CFTR exposed to calpain are identical. However, in intact cells the rate of digestion of the mutated 170 kDa channel protein was considerably higher (see Fig. 4B). To explain this apparent discrepancy, we analyzed the effect of association of HSP90 to F508del-CFTR on its susceptibility to calpain digestion [24].

By means of density gradient centrifugation of total membrane fraction from FRT cells expressing F508del-CFTR and JA3 cells, the plasma membranes, containing exclusively the 170 kDa CFTR forms, were separated from the low density membrane fraction, containing the 100 kDa split form (see Fig. 2). Each solubilised membrane fraction, containing comparable amounts of 170 and 100 kDa CFTR, was then incubated with increasing concentrations of HSP90 and the association of CFTR with the chaperone was determined by immunoprecipitation. It was observed that (Fig. 5A), while the maximal association between HSP90 and wild type 170 kDa CFTR occurred at a concentration of the chaperone around 0.9 nM, the interaction with 170 kDa F508del-CFTR became detectable only at 3 nM HSP90 and was maximal at 6 nM. Thus, Phe^{508} deletion promoted a decrease in the affinity of 170 kDa CFTR for the chaperone, approximately corresponding to one order of magnitude. The same analysis, carried out on solubilized membranes containing the split 100 kDa CFTR forms, revealed that binding to HSP90 was not affected by the Phe^{508} deletion (Fig. 5B). To establish if the different affinity to the chaperone of the wild type and 170 kDa F508del-CFTR could thus be responsible for their different sensitivity to calpain digestion, the two CFTR forms were incubated with the protease in the presence of HSP90 (see Fig. 5C). Whereas, the digestion of wild type 170 kDa CFTR was completely prevented in the presence of 0.9 nM HSP90, the 170 kDa F508del-CFTR digestion was approximately 40% reduced at 3 nM HSP90 and 80% reduced at 6 nM. These results are in agreement with those previously shown in Fig. 5A, indicating that the association to HSP90 of the 170 kDa mutated channel protein is significantly reduced. As expected, the two split 100 kDa CFTR forms were both protected from calpain digestion in the presence of 0.9 nM HSP90 (Fig. 5D). The protection of the protein channel, resulting from its association to HSP90, suggests a novel role of the chaperone in the preservation of functional CFTR at the plasma membrane.

3.5. Involvement of different proteolytic systems on the level and cellular localization of CFTR

In order to establish how the calpain-mediated degradation of F508del-CFTR could affect the level of plasma membrane chloride channel, growing FRT cells expressing F508del-CFTR were exposed to nontoxic amounts of C.I.2 [38]. For comparison, FRT cells were also incubated with MG132, an inhibitor of proteasome activity. As shown in Fig. 6, following 24 h (approximately two cell cycles) of exposure to C.I.2, the level of the 170 kDa form at the plasma membrane was 8- to 10-fold augmented, clearly demonstrating that the reduction of intracellular calpain activity favoured an increase in the level of native 170 kDa CFTR recovered in its correct functional localization. Similar results were obtained with other calpain inhibitors (C.I.1 and PD151746), indicating that their effect was not due to unspecific metabolic alterations (data not shown). It is interesting to note that, in these conditions in which calpain activity was reduced, the 100 kDa split form became undetectable, thus confirming previous results showing that the removal of the split CFTR fragment requires the endosome–lysosome pathway rather than the Ca^{2+}-dependent protease activity [9,15,24]. In the presence of MG132, no appreciable changes in the level of plasma membrane F508del-CFTR were observed and no accumulation of the 100 kDa form occurred. An increased fluorescence diffused throughout the cytoplasm indicated that these conditions favoured an accumulation of the 150 kDa CFTR form that was trapped within the cell and that proteasomal activity was not involved, in our experimental conditions, in the removal of native F508del-CFTR from plasma membranes.

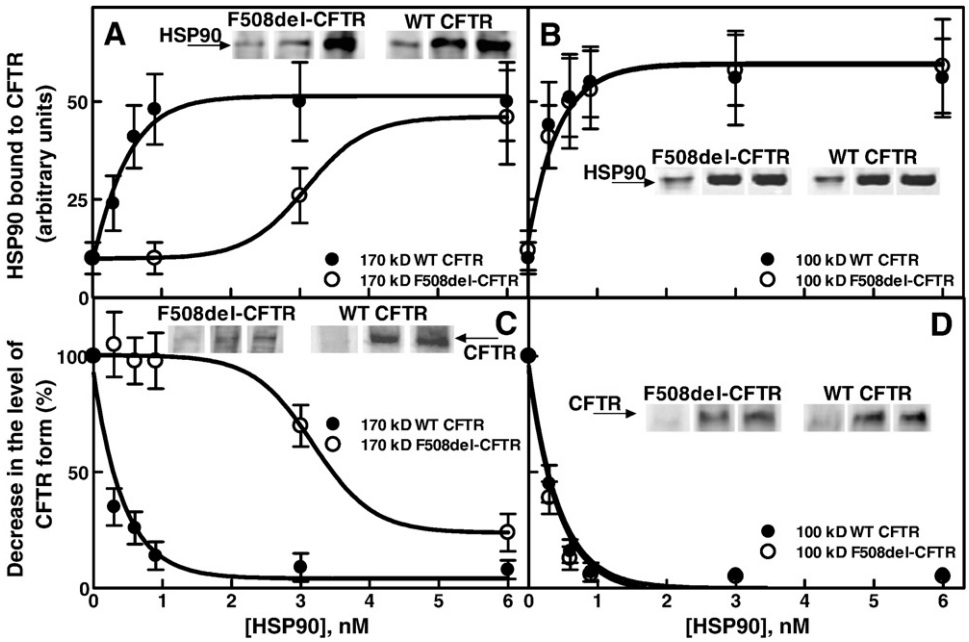

Fig. 5. Interactions of WT and F508del-CFTR forms with HSP90. (A) Plasma membrane samples, isolated from JA3 cells and FRT cells expressing F508del-CFTR, containing comparable amounts of 170 kDa WT and 170 kDa F508del-CFTR, evaluated on the basis of the intensity of their immunoreactive bands, were solubilized in 1% Triton® X-100 and the indicated amounts of HSP90 were added to the suspensions. Immunoprecipitation was carried out using anti-CFTR mAb M3A7 (see Materials and methods). HSP90 co-immunoprecipitated with 170 kDa CFTR forms was detected by immunoblotting using the specific antibody and the immunoreactive bands were quantified as described in Materials and methods. The values are reported as the arithmetic mean ± SD of three different experiments. (B) The same immunoprecipitation described in (A) was performed using intracellular membranes containing comparable amounts of 100 kDa WT and 100 kDa F508del-CFTR. HSP90 co-immunoprecipitated with 100 kDa CFTR forms was detected by immunoblotting using the specific antibody and the immunoreactive bands were quantified and the values are reported as the arithmetic mean ± SD of three different experiments. Aliquots (20 μg) of plasma (C) and intracellular (D) membrane fraction, isolated from JA3 cells and FRT cells expressing F508del-CFTR, were incubated at 37 °C with 1 unit of calpain in the presence of the indicated amounts of HSP90. After 10 min, the incubations were stopped by the addition of Laemmli SDS-PAGE loading solution. The samples were then incubated at 37 °C for 30 min and submitted to 6% SDS-PAGE, followed by immunoblotting. CFTR was detected using mAb M3A7. The immunoreactive bands corresponding to 170 (C) and 100 (D) kDa CFTR forms were quantified and the values are reported as the arithmetic mean ± SD of three different experiments. The images inserted in each panel are representative of each experiment performed using respectively 0, 0.9 and 6 nM HSP90.

Several reports have indicated that small molecules correctors, such as Corr-4a, enhance maturation of F508del-CFTR by recovering the interaction between the two halves of the channel [17–20]. In order to explore if the effect of these compounds implied mechanisms involving calpain activity, FRT cells expressing F508del-CFTR were exposed to Corr-4a. In these conditions, a significant increase in the CFTR fluorescence diffused in cytoplasm was presumably attributable to the accumulation of large amounts of immature 150 kDa CFTR form. Since the level of 170 kDa CFTR at the plasma membrane was increased less than 2-fold, it can be concluded that the Corr-4a promoted a refolding of F508del-CFTR predominantly accumulated in a still immature channel form unable to reach the plasma membrane.

4. Discussion

The mechanisms responsible for the regulation of CFTR trafficking and for the delivery of the mature channel form at the apical membrane level involve a multistep process which includes synthesis, correct protein folding and maturation [4,5,39]. Furthermore, in order to prevent cellular accumulation of misfolded proteins, nonnative CFTR molecules are degraded by the ubiquitin proteasome system as part of the ER-associated quality control [11,40]. It has also been proposed that an additional peripheral quality control can remove, via lysosomal degradation, unfolded plasma membrane-bound CFTR molecules that have escaped the ER control [12–15]. Both mechanisms are thus operating in the recognition and removal of misfolded molecules in the course of the maturation of both the wild type and F508del-CFTR, the most common mutation of the channel protein occurring in CF patients characterized by deletion of Phe^{508}. However, since the wild type CFTR molecules undergo predominantly a correct folding, only a small fraction of unfolded molecules are degraded, while most of them reach the plasma membrane. In the case of F508del-CFTR, due to a folding defect, most of the protein is degraded before reaching its functional location [41–43]. Different experimental approaches including inhibitors of ERAD or exposure to low temperature (27 °C), being the conformational defect temperature-sensitive, or alternatively treatment with small-molecule correctors, were only partially effective in rescuing functional F508del-CFTR which however, resulted to be highly instable and rapidly degraded at 37 °C.

We have previously reported that, in growing cells, plasma membrane CFTR, presumably as a part of its normal turnover, undergoes degradation by calpain into a limited number of products internalized into endosomal vesicles [24]. The experimental evidences were the following: in the presence of calpain inhibitors no degradation of CFTR occurred and no degradative products were accumulated. Moreover, by immunoblotting analysis, using an antibody directed against the C-terminal region of CFTR, both in vitro and in vivo digestions were monitored by the appearance of a 100 kDa fragment. Based on the size of this fragment, the calpain cleavage appeared to occur in a region between the NBD1 region and the regulatory domain. Since in nondenaturating conditions both native and cleaved CFTR form showed identical mass–charge ratio, the proteolytic event did not promote the release of fragments from cytoplasmic facing CFTR domain. Such 100 kDa product could be also accumulated following exposure of native CFTR to trypsin [24], indicating that CFTR contains a highly protease sensitive sequence, possibly involved in determining the endocytic internalization. Furthermore, the presence of high amounts of the 100 kDa fragment was observed in PBMC of normal human subjects indicating that CFTR degradation by calpain is a physiologically occurring process [24].

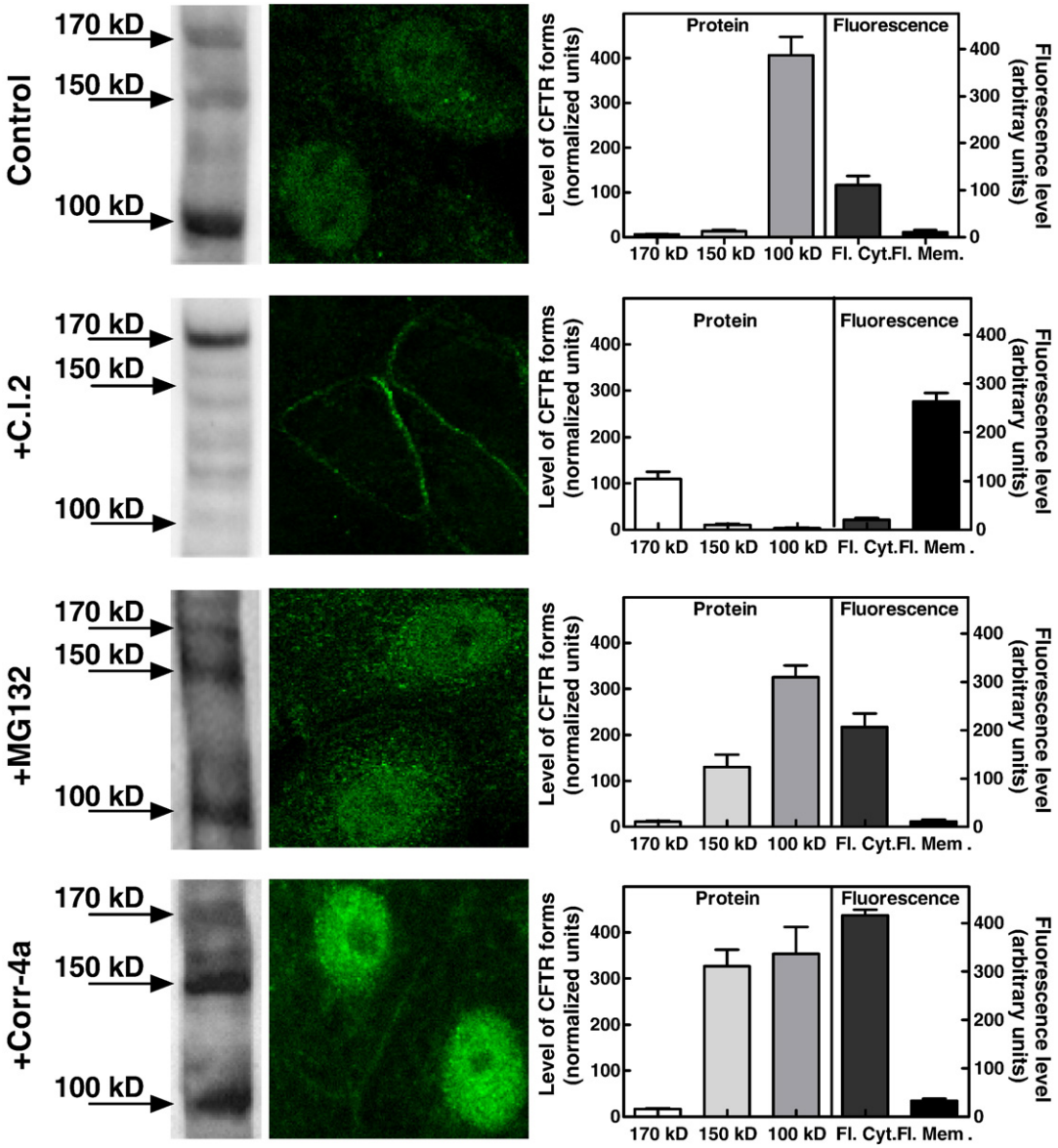

Fig. 6. Effect of different proteolytic systems on the level and localization of F508del-CFTR. FRT cells expressing F508del-CFTR were grown for 24 h in the absence (control) or presence of 1 μM C.I.2 (+C.I.2), or 1 μM MG132 (+MG132), or 5 μM Corr-4a (+Corr-4a). Aliquots (50×10^4 cells) were lysed in 100 μl of Laemmli SDS-PAGE loading solution, incubated at 37 °C for 30 min and 30 μl were submitted to 6% SDS-PAGE, followed by immunoblotting. The arrows indicate the 170, 150 and 100 kDa CFTR forms detected using M3A7 mAb and the immunoreactive bands were quantified as described in Materials and methods. The values are reported as the arithmetic mean ± SD of three different experiments. The images are representative of three different experiments. For each treatment, intracellular CFTR localization was determined by confocal microscopy using M3A7 mAb and fluorescence level was quantified (see Materials and methods) and reported as the arithmetic mean ± SD of three different experiments. The images are representative of three different experiments.

In the present paper we have tested the hypothesis according to which a selective proteolysis of F508del-CFTR occurring at the plasma membrane could be responsible for the very low level of the mutated chloride channel, normally detectable in cells of CF patients. A first support to this hypothesis was provided by the findings, herewith reported, that in lymphocytes from CF patients the 100 kDa species were the almost only detectable form of CFTR, being the 170 kDa form present in negligible amounts.

We have also established that F508del-CFTR is cleaved by calpain both in reconstructed system as well as in Ca^{2+}-loaded cells and that digestion produces the same fragments that are generated following degradation by the protease of the wild type channel protein form. However, in spite of a similar in vitro sensitivity to calpain of the wild type and of the mutated CFTR, we have observed that, in intact Ca^{2+}-loaded cells, the 170 kDa F508del-CFTR was 3- to 5-fold more sensitive to digestion by the protease and thus degraded at a much faster rate. This higher susceptibility to calpain was due to a lower capacity of the 170 kDa mutated channel protein to interact with HSP90. Following the cleavage, the affinity of F508del-CFTR for the chaperone was fully recovered, thus explaining the intracellular constitutive presence of high amounts of the 100 kDa form. Consistently, in cells expressing F508del-CFTR, the cleaved channel form, identified by the presence of the 100 kDa fragment, was pre-existent in larger amounts than the 170 kDa form also before Ca^{2+} loading and, furthermore, inhibition of intracellular calpain activity during cell growth, resulted in a dramatic increase (10-fold) of the 170 kDa form with the concomitant complete disappearance of the cleaved CFTR form. Thus, calpain inhibition promotes the rescue of significant amounts of the mutated CFTR at plasma membrane. Parallel experiments performed using Corr-4a demonstrated that only a small amount of 170 kDa F508del-CFTR reached the plasma membrane, whereas a large quantity of CFTR

with a mass of approximately 150 kDa was accumulated in a cytoplasmic localization. This large accumulation of the 150 kDa CFTR is consistent with our previous observations indicating that this form is resistant to intracellular calpain digestion [24]. Furthermore, since in these conditions the level of the 100 kDa remained unchanged, it can be concluded that C.I.2 and Corr-4a operate at different levels since the former promotes preferentially the accumulation of CFTR at the plasma membrane and the latter that of the channel protein in the course of its maturation at ER. The role of calpain in the regulation of CFTR level at plasma membrane is further indicated by the direct relationship observed between the extent of CFTR degradation and intracellular activation of the protease.

Recent observations from different laboratories [44–46] indicate that a number of membrane-associated protein channels are targets of proteases, including calpains. Although, at present, a general role of such proteolysis on the functions of these channels cannot be defined, it can be postulated that this limited proteolysis might be involved in the regulation of the channels levels at the plasma membrane as well as in the control of their transport efficiency.

In summary, our observations demonstrate that an enhanced limited proteolysis by calpain of plasma membrane F508del-CFTR, sustained by a decreased binding capacity for HSP90, contributes in determining the very low level of the mutated channel at its functional location. This degradation could be further enhanced by the alteration of Ca^{2+} homeostasis reported to occur in CF epithelial airway cells [23,47–50].

Altogether these findings suggest that the Ca^{2+}-dependent proteolysis can be regarded as a target for new therapeutic approaches directed to ameliorate the life quality of CF patients. They can also represent additional evidence in favour of a membrane localized activity of calpain directed to degradation of transmembrane proteins both in physiological and in pathological conditions such as those herewith considered and more generally characterized in the so called conformational diseases.

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