



## The role of calpain in the regulation of CFTR turnover

Monica Aversa, Roberto Stifanese, Raffaella Grosso, Marco Pedrazzi, Roberta de Tullio, Franca Salamino, Sandro Pontremoli, Edon Melloni

### ► To cite this version:

Monica Aversa, Roberto Stifanese, Raffaella Grosso, Marco Pedrazzi, Roberta de Tullio, et al.. The role of calpain in the regulation of CFTR turnover. *Biochemical Journal*, 2010, 430 (2), pp.255-263. 10.1042/BJ20100344 . hal-00509882

**HAL Id: hal-00509882**

**<https://hal.science/hal-00509882>**

Submitted on 17 Aug 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**The role of Calpain in the Regulation of CFTR Turnover**

**Monica Averna, Roberto Stifanese, Raffaella Grosso, Marco Pedrazzi, Roberta De Tullio, Franca Salamino, Sandro Pontremoli and Edon Melloni**

Department of Experimental Medicine (DIMES) - Biochemistry Section, and Centre of Excellence for Biomedical Research (CEBR), University of Genoa, Viale Benedetto XV, 1-16132 Genoa, Italy

**Correspondence to:** Prof. Edon Melloni, University of Genoa, DIMES-Biochemistry Section, Viale Benedetto XV, 1 - 16132 Genoa - Tel: 39-010-3538128; Fax: 39-010-518343; e-mail: [melloni@unige.it](mailto:melloni@unige.it)

**Running title:** *CFTR digestion by calpain*

**Keywords:**  $\text{Ca}^{2+}$ -dependent proteolysis; intracellular calpain regulation; CFTR; HSP90; lymphocytes; NMDA receptor.

## Summary

The level of mature native 170 kD at plasma membrane is under the control of a selective proteolysis catalyzed by calpain. The product of this limited digestion, consisting of discrete fragments still associated by strong interactions, is removed from the plasma membrane and internalized in vesicles addressed to an additional degradation. This process can be monitored by the accumulation of a 100 kD fragment in growing human leukaemic T cell line and in human circulating lymphocytes. In reconstructed systems and in intact cells, the conversion of native CFTR into 100 kD fragment linearly correlated to calpain activation and was prevented by addition of synthetic calpain inhibitors. Reduction in  $\text{Ca}^{2+}$  influx by blocking the NMDA receptor  $\text{Ca}^{2+}$  channel inhibited conversion of native 170 kD into 100 kD fragment, whereas the endosome acidification blocker promoted accumulation of the 100 kD CFTR digested form. An important role in calpain-mediated turnover of CFTR is exerted by HSP90, which, associating to the protein channel, modulates the degradative effect of calpain through a selective protection.

Altogether these results indicate that CFTR turnover is initiated by calpain activation, induced by an increased  $\text{Ca}^{2+}$  influx and, following internalization of the cleaved channel protein, it is completed by the lysosomal proteases.

These findings are providing new insights in the understanding of the molecular mechanisms responsible for the defective functions of ion channels in human pathologies.

## Introduction

It is generally considered that in physiological conditions a regulated intracellular trafficking controls the expression of native functionally active cystic fibrosis transmembrane conductance regulator (CFTR), at the apical membrane level [1-5]. This assumption implies that properly folded nascent CFTR escaping proteasomal degradation, leaves the endoplasmic reticulum (ER) and travels directly to the plasma membrane. However, a complete understanding of the integrated CFTR trafficking mechanisms is still to be completely defined. A critical question concerns the use of overexpressing systems or non physiological experimental conditions. Also the problem about how CFTR is localized at the plasma membrane and regarding the precise contribution of its PDZ (PSD-95, Discs large, Zonula occludens-1) anchoring domain, as an apical membrane retention motif, needs to be clearly established, also with respect to CFTR endocytic recycling [6, 7]. Moreover, the action of cytoplasmic proteases, postulated to participate to CFTR degradation in addition to the proteasomal-ubiquitin pathway [8, 9], needs to be more precisely characterized in its molecular and physiological properties [10].

To provide new insights in the intracellular regulation of CFTR trafficking, we have investigated if calpain, the  $\text{Ca}^{2+}$ -dependent protease [11, 12], was involved in this process. These studies were also suggested by the observations of a functional relationship between CFTR and  $\text{Ca}^{2+}$  homeostasis and by the indication that  $[\text{Ca}^{2+}]_i$  is elevated in epithelial airway cells of cystic fibrosis (CF) patients [13-15].

Furthermore, even though there is no direct evolutionary relationship between CFTR and other transmembrane ion channels, including glutamate receptors, it must be considered that several of these proteins share some common molecular features such as the ability to bind PDZ domains and the specific vulnerability to calpain cleavage [5, 16-20]. Although no clear information on the physiological role of such digestion is available, it is noteworthy to recall that calpain normally cleaves a very limited number of peptide bonds on its substrates, thus producing discrete forms that can be easily accumulated [11, 12]. The biochemical significance of this conservative proteolysis is the rapid and irreversible generation of new protein forms with modified functional properties.

In the present study, using isolated membranes or intact cells, we demonstrate that wild type CFTR is a highly susceptible calpain substrate. Intracellular proteolysis of CFTR by calpain proceeds through the accumulation of discrete CFTR fragments which are then internalized in endosomes. This is an indication of a limited proteolytic cleavage, occurring on the inner surface of the plasma membrane, whose possible physiological importance is supported by the identification of the CFTR

fragments in circulating human lymphocytes and by the finding of a direct relationship between the accumulation of these fragments and the calpain activity.

We propose that, in addition to the well established proteasomal degradation and to that related to the endocytic recycling, CFTR undergoes a further proteolytic down-regulation sustained by the activity of calpain. The peculiar property of this mechanism is its occurrence at the plasma membrane favouring endocytosis of the modified channel.

Altogether these findings, not only provide new evidences in support of a novel physiological role of calpain in the regulation of the turnover of transmembrane ion channels, but also may be indicative of a new target of potential therapeutic implications in CF pathology.

## Experimental

### *Materials*

Nutrient mixture F-12 Coon's modification, RPMI1640, leupeptin, calpain inhibitor 2 (C.I.2), calcium ionophore A23187, Triton<sup>®</sup> X-100, and trypsin 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. *t*-Boc-Leu-Met-CMAC fluorogenic calpain substrate was purchased from Molecular Probes (Invitrogen). 4-(2-aminoethyl) benzenesulfonylfluoride (AEBSF) and bafilomycin A1 were obtained from Calbiochem. Peroxidase-conjugated secondary antibody was obtained from Cell Signaling. MK801 (dizolcipine) was from Merk Sharp and Dohme (Hoddesdon, UK). ECL ADVANCE<sup>®</sup> Detection System, Ficoll-Pacque Plus, and peroxidase-conjugated secondary antibody were obtained from GE Healthcare. Human erythrocyte calpain was isolated and assayed as reported in [21]. One unit of calpain activity is defined as the amount that releases 1 nMol/h of free  $\alpha$ -amino groups under the specified conditions [22]. HSP90 was purified from rat brain as reported in [23].

### *Monoclonal antibodies*

Anti-CFTR antibody (clone M3A7) was purchased from Millipore. Anti-CFTR antibody (clone 24-1) was purchased from R&D systems. Anti-HSP90 antibody was obtained from BD Biosciences.

### *Methods*

#### *Cell Culture*

Fisher rat thyroid cell line overexpressing human CFTR (FRT cells) was cultured at 37°C (5% CO<sub>2</sub>) 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 cells not expressing human CFTR (FRT not-transfected cells) were cultured in the same medium of FRT cells in the absence of zeocin. FRT cell lines were kindly provided by Dr. L.J. Galletta, Molecular Genetics Laboratory, Giannina Gaslini Institute Genova, Italy. Human leukaemic T cell line (JA3 cells) was cultured at 37°C (5% CO<sub>2</sub>) with RPMI1640 growth medium, containing 10% FBS, 10 U/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine (medium A). JA3 cells overexpressing Cast48 (JA3-cast) [24] were cultured in medium A containing 1 mg/ml geneticin.

#### *Isolation of total membrane fraction*

FRT cells, overexpressing CFTR, JA3 cells and human circulating lymphocytes, purified as previously reported [25], were collected and washed three times with ice-cold phosphate buffered saline (PBS) solution.

Total membrane fraction was isolated following lysis of  $25 \times 10^6$  cells in 1ml 1mM EDTA, pH 7.0 (buffer A) containing 5 µg leupeptin and 10 µg AEBSF by three cycles of freezing and thawing, followed by centrifugation at 60,000 x 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.

#### *Immunoprecipitation and immunoblotting*

Total membrane fraction, prepared as described above from FRT cells, or incubated with 1 unit of calpain in the presence of 1mM CaCl<sub>2</sub> for 10 min at 37°C, was solubilized in 300 µl of buffer A containing 0.15 M NaCl and 0.1% Triton<sup>®</sup> X-100. Aliquots of HSP90 purified from rat brain [23]

were then added to the membrane suspension. The mixtures, pre-treated with protein G-Sepharose, were incubated over night with 2 µg of anti-HSP90 mAb or with 2 µg of anti-CFTR antibody (clone M3A7) at 4°C. Protein G-Sepharose was then added to the samples and incubated for an additional 1 h in continuous and gently 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 6 % SDS-PAGE [26]. Proteins were then transferred to nitrocellulose membranes (Bio-Rad) by electroblotting [27] and probed with anti-CFTR antibody (clone M3A7) or with anti-HSP90 mAb, followed by a peroxidase-conjugated secondary antibody as described in [28]. The immunoreactive material was developed with ECL ADVANCE<sup>®</sup> detection system, detected with a Bio-Rad Chemi Doc XRS apparatus and quantified using the Quantity One 4.6.1 software (Bio-Rad).

#### *Confocal microscopy imaging and fluorescence quantification*

FRT cells, grown on glass slides (10 x 10<sup>4</sup> cells), were fixed and permeabilized by the Triton/paraformaldehyde method, as described in [29]. Cells were treated with 10 µg/ml CFTR antibody (M3A7) diluted in PBS solution, containing 5% (v/v) foetal bovine serum. After incubation for 3 h at room temperature, cells were washed three times with PBS solution and treated with 4 µg/ml chicken anti-mouse 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. Images were collected by a Bio-Rad MRC1024 confocal microscopy, using a 60x 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 [30].

#### *Assay of intracellular calpain activity*

Calpain activity was detected in JA3 intact cells following the procedure described in [24]. Briefly JA3 cells were collected, washed three times in PBS solution and incubated for 20 min at 37°C in 10 mM Hepes pH 7.4, containing 0.14 M NaCl, 5 mM KCl, 5 mM Glucose (buffer B) containing 50 µM t-Boc-Leu-Met-CMAC fluorogenic calpain substrate. Cells were then washed twice with buffer B in order to remove substrate excess. The pellets were suspended, at a concentration of 1 x 10<sup>6</sup> cells/ml, in buffer B containing 0.1 mM CaCl<sub>2</sub>. Aliquots of cells (0.2 ml) were transferred in a 96 wells plate and the fluorescence emission was continuously monitored at 37°C with a Mithras LB 940 plate reader (Berthold Technologies). Calpain activity was stimulated by the addition of 0.1 µM calcium ionophore A23187. The excitation/emission wavelengths were 355/485 nm, respectively.

#### *Native electrophoresis*

Total membrane fraction, prepared from FRT cells as described above, were solubilized in 100 µl of 0.35 M Tris/HCl pH 9.0 (buffer C), containing 0.5 M KCl and 0.1% Triton<sup>®</sup> X-100. After for 2 hours at 4°C loading buffer (buffer C containing 10% glycerol) was added. The samples were loaded on to a 0.8% agarose gel, after the electrophoretic run, were transferred to nitrocellulose membranes (Bio-Rad) by electroblotting [27] and probed with anti-CFTR antibody (clone M3A7) followed by a peroxidase-conjugated secondary antibody as described in [28]. The immunoreactive material was developed with ECL ADVANCE<sup>®</sup> detection system, detected with a Bio-Rad Chemi Doc XRS apparatus.

#### *Isolation of plasma and intracellular membranes*

Total membrane fraction from JA3 cells was fractionated on a discontinuous sucrose density gradient [10, 20, 30, 50 % (w/v)]. After centrifugation at 60,000 x g for 30 min, membranes were separated in two fractions layered between the 10-20% and 30-50% steps. Membrane fractions were separately collected, suspended in 0.2 ml buffer A each, and 5'-Nucleotidase (5'-NT) activity was assayed [31].

## **Results**

#### *CFTR forms in FRT, JA3 cells and human circulating lymphocytes.*

Immunoblot analysis of total membrane proteins isolated from CFTR overexpressing FRT cells and from human leukemic T cell line (JA3 cells), using an antibody directed to the C-terminal region of



CFTR, revealed a second band with a Mr of 100 kD in addition to the native 170 kD (Fig. 1). The level of this low Mr CFTR species represented less than 1% in FRT cells (Fig. 1A), whereas in JA3 cells its level exceeded 1.5-1.7-fold that of the native 170 kD form (Fig. 1B). In both cell lines, it was also detected a small amount of a third 150 kD CFTR band, normally considered an ER associated form in the course of its maturation (Fig. 1) [32]. The 100 kD protein was also detected by using a different primary antibody recognizing the C-terminal region of CFTR as well, followed by a different secondary antibody. Moreover, this low Mr CFTR band was identified in human lymphocytes membranes in an amount approximately 2-fold higher as compared to the native form (Fig. 1C). The value of the Mr of this CFTR form could be due to a post-translational cleavage occurring after maturation of the protein channel. On the basis of this assumption and of previous observations showing that the cytosolic CFTR domain, expressed in reticulocyte lysates, is degraded by a soluble still unidentified protease [10], we have studied the involvement of the  $\text{Ca}^{2+}$ -dependent proteolytic system in the formation of low Mr CFTR forms.

#### *Digestion of CFTR by calpain.*

Our studies were first directed to establish if CFTR was a substrate of calpain. Accordingly, total membrane fraction, isolated from CFTR overexpressing FRT cells was exposed to the purified protease. As shown in Fig. 2A, the native 170 kD CFTR protein band progressively disappeared with the concomitant accumulation of a 100 kD protein band in an amount corresponding to 70% of the original level of the 170 kD protein band (Fig. 2A, lower panel). Such incomplete recovery could be due to a further degradation of the 100 kD protein occurring at slowest rate, not detectable in these conditions. Following centrifugation of the incubation mixture, the digested CFTR was still recovered in the membrane fraction, indicating that calpain cleavage did not cause the release of the splitted channel protein from the membrane structures. As previously reported [33], a CFTR digestion product with similar Mr has been obtained following exposure of isolated membranes to trypsin (Fig. 2A). This was indicating that, in CFTR cytosolic domain, a segment highly susceptible to digestion by soluble proteases is present. To extend these observations to cells constitutively expressing CFTR, membrane fractions from JA3 cells were isolated and subsequently exposed to purified calpain. In these conditions (Fig. 2B), the 170 kD CFTR as well as the already present 100 kD form progressively decreased, although at a consistently different rate (Fig. 2B, lower panel). The lower susceptibility to calpain digestion of the 100 kD segment could account for its accumulation. CFTR digestion did not occur in the absence of the protease and was prevented by the addition of the protease inhibitor C.I.2 (data not shown).

It is well known that calpain catalyzes the hydrolysis of a very limited number of peptide bonds in its target proteins, thereby promoting conservative modifications of its substrates [11, 12]. In accordance, the high levels of the 100 kD CFTR are consistent with these calpain catalytic properties capable to promote the cleavage of the CFTR polypeptide chain in discrete segments remaining still associated on the membranes, as a result of the strong interactions between the channel domains [34]. To verify this hypothesis, membrane samples from FRT cells, containing native or calpain-digested CFTR, were solubilized in 0.1% Triton X-100 and submitted to electrophoresis in non denaturing conditions followed by immunoblotting. As shown in Fig. 2C, native and digested CFTR forms showed identical electrophoretic mobility, indicating that calpain-mediated cleavage of CFTR did not alter the charge/mass ratio of the protein, producing still associated discrete portions of which the 100 kD fragment represents the marker.

#### *Changes in CFTR cellular localization following digestion by calpain*

To define if the calpain-mediated cleavage of CFTR occurred also in intact cells, FRT cells were loaded with  $\text{Ca}^{2+}$  to promote intracellular calpain activation [11]. As shown in Fig. 3A, native 170 kD CFTR band disappeared almost completely being replaced by the 100 kD form. When  $\text{Ca}^{2+}$  loading was preceded by exposure of FRT cells to C.I.2, accumulation of the 100 kD band was completely prevented and the native CFTR band was fully preserved (Fig. 3A and B).

By confocal microscope imaging, it was established that, in the course of the proteolytic conversion of CFTR in  $\text{Ca}^{2+}$ -loaded cells, the protein fluorescence, originally detected exclusively on plasma

membrane (Fig. 3C, left panel), appeared now to be distributed as spots within the cytosolic compartment (Fig. 3C, middle panel). None of these changes occurred in the presence of C.I.2 (Fig. 3C, right panel).

Also in JA3 cells, following  $\text{Ca}^{2+}$  loading, both 170 kD and 100 kD protein bands significantly decrease (Fig. 4A), whereas, cell pre-treatment with C.I.2 completely prevented proteolysis.

The constitutively presence of 100 kD CFTR segment in JA3 cells indicated that calpain-mediated CFTR cleavage occurred spontaneously in growing cells, in which a mild activation of the protease was previously demonstrated [24]. Thus, the correlation between the level of the 100 kD protein band, the marker of CFTR limited proteolysis, and the potential intracellular expression of calpain activity was explored. In JA3 cells grown for two cell cycles in the presence of C.I.2 or in JA3 cells expressing high levels of calpastatin (JA3-cast), the intracellular calpain activity was reduced more than 80% (Fig. 4B) and concomitantly the level of the 100 kD CFTR segment became almost undetectable (Fig. 4B, upper panel). These results indicated that the formation of the CFTR segment was directly related to the extent of calpain activation (Fig. 4B, lower panel). Following reduction in intracellular calpain activity, the level of native CFTR was largely increased becoming from 3 to 7-fold higher as compared to control cells. The parallel disappearance of the 100 kD segment, occurring in these conditions, could only be explained by assuming that other proteolytic mechanisms were responsible for completion of the CFTR turnover initiated by calpain.

#### *Intracellular processing of calpain modified CFTR*

To further define the role of the initial conservative proteolysis of CFTR by calpain, we have studied the sequential steps leading from CFTR internalization to its complete degradation. For this purpose, total membrane fraction from JA3 cells was fractionated by centrifugation on a four steps sucrose density gradient (Fig. 5A). Two fractions were obtained: in the first one, containing low density vesicles, only the 100 kD CFTR species were detected, in the second one, containing high density vesicles characterized by the plasma membrane marker 5'-NT activity, the native 170 kD CFTR was detected. Moreover, in JA3 cells overexpressing calpastatin, native CFTR was identified in large amounts and exclusively in high density vesicles derived from plasma membrane (Fig. 5B). No appreciable CFTR forms were detectable on the low density vesicles. These findings support previous observations (see Fig. 4) indicating that calpain digests native CFTR at the plasma membrane and that, following the cleavage, the splitted channel is internalized in endocytic vesicles. The absence of native 170 kD CFTR in low density vesicles further suggests that the recycling of the channel protein is not significantly related to the formation of the 100 kD fragment. Although our observations indicated that the 100 kD fragment was generated by cleavage of the native 170 kD form, an additional source of the modified channel could be the 150 kD CFTR retained in ER. To verify this possibility, we have analyzed the susceptibility of the 150 kD CFTR to calpain digestion, both in isolated membranes and in  $\text{Ca}^{2+}$ -loaded cells. As shown in Fig. 6A, the small amount of 150 kD CFTR, detectable in FRT cell membranes, was equally sensitive to calpain as the native form. However, in  $\text{Ca}^{2+}$ -loaded cells (Fig. 6B), at difference with the native 170 kD CFTR extensively degraded, the 150 kD form remained almost unaffected.

To provide further information on the events initiating the calpain-mediated conversion of CFTR, growing JA3 cells expressing *N*-methyl-D-aspartic acid (NMDA) receptor (data not shown), a glutamate triggered  $\text{Ca}^{2+}$  channel, were treated with MK801, a selective non-competitive antagonist of the channel [35]. As shown in Fig. 7, in JA3 cells growing for two cycles in the presence of MK801, the level of native 170 kD CFTR was approximately 3-fold increased in respect to control, while the 100 kD fragment completely disappeared. Thus, reducing the  $\text{Ca}^{2+}$  influx during cell growth, digestion of CFTR at plasma membrane no longer occurs, in agreement with the effect obtained following intracellular calpain inhibition. To explain the reason for the disappearance of the cleaved CFTR, in conditions in which activation of calpain was inhibited, JA3 cells were exposed to bafilomycin, known to abolish the endocytic acidification [36]. In the presence of non toxic amounts of this compound, the level of native CFTR was only poorly reduced, whereas the level of the 100 kD segment was more than 3-fold increased. In accordance with previous reports

[37], exposure of growing cells to the proteasoma inhibitor MG132 did not affect the level of both native and digested CFTR indicating that the ubiquitin-proteasoma pathway is not involved in the cleavage of CFTR at plasma membrane (data not shown).

#### *Effect of HSP90 on the CFTR susceptibility to calpain by HSP90*

Previous reports have suggested that many chaperones, including HSP90, are involved in the maturation of CFTR inside ER [33, 38]. We have recently demonstrated [20, 23] that HSP90 is active also in the cytoplasmic compartment in protecting native and correctly folded NO synthase isozymes from digestion by calpain. On the basis of these observations, we explored if HSP90 could interact with native CFTR as well as with the 100 kD segment. As shown in Fig. 8A, immunoprecipitation experiments revealed that HSP90 associated with both native and cleaved CFTR form. The same experiments were also performed using FRT cells not expressing CFTR. No cross reactivity was detected by anti-CFTR antibody as well as HSP90 was not co-immunoprecipitated (data not shown). In order to determine if this interaction could affect the sensitivity of CFTR to calpain digestion, membrane preparations containing the native (Fig. 8B) or the cleaved (Fig. 8C) CFTR forms were exposed to purified calpain in the absence or presence of purified HSP90 [23]. The addition of the chaperone reduced more than 50% the rate of digestion of the native channel and prevented almost completely the degradation of the 100 kD fragment. Replacement of HSP90 with the same amount of BSA did not prevent CFTR digestion. Calpain-induced conversion of the native into the digested CFTR form appears to induce a conformational change increasing the affinity of the protein for HSP90. These results, besides suggesting a regulatory function of HSP90 on CFTR susceptibility to proteolysis, are also indicating a novel role of the chaperone in the turnover of native CFTR molecules.

To establish if digestion of CFTR by calpain and the accompanying changes in cellular localization (see Fig. 3) could also be affected by HSP90, FRT cells were loaded with  $\text{Ca}^{2+}$  in the absence or presence of geldanamycin (GA), an inhibitor of HSP90 ATPase activity [39, 40]. Addition of GA, to  $\text{Ca}^{2+}$ -enriched cells, promoted the almost complete disappearance of all CFTR forms from plasma membrane as well as from cytoplasmic vesicles (Fig. 9). If cells were also treated with C.I.2, disappearance and redistribution of CFTR was completely prevented, further supporting that calpain-mediated proteolysis is the primary event in CFTR turnover.

#### **Discussion**

Intracellular degradation of CFTR is an important mechanism in determining the level of expression of CFTR at the apical plasma membrane [6-8, 41-43]. Thus, in addition to the involvement of proteasome in ER and endosomal recycling [44], experimental evidences indicate a possible role of cytoplasmic cystein-protease in the digestion of CFTR [10]. These findings, obtained in an *in vitro* biochemical assay using an isolated cytoplasmic CFTR domain, have also suggested a selective susceptibility to degradation of  $\Delta\text{F508}$  CFTR by this unidentified protease.

In the present paper we demonstrate that CFTR is a highly susceptible substrate of calpain, which cleaves the single polypeptide chain of native 170 kD form in discrete segments. This process has been monitored by measuring the formation of a 100 kD segment still associated to cell membranes. Since in native conditions the cleaved CFTR showed electrophoretic properties identical to those of the intact protein, calpain cleavage must occur at a very limited number of peptide bonds and the generated fragments remain associated by strong interactions between domains and insertion into the membrane bilayer. This limited proteolysis occurs almost exclusively at the inner surface of plasma membrane in a region between the first nucleotide binding domain and the regulatory domain. This is supported by the observation that native CFTR is predominantly localized at plasma membrane [45], and that, in conditions of intracellular calpain inhibition, this CFTR form is not recovered in appreciable amounts in internal recycling vesicles. The immature 150 kD CFTR form, localized at the ER membranes, does not seem to be an alternative source of the cleaved CFTR, since it was resistant to calpain in  $\text{Ca}^{2+}$ -loaded cells.

Of particular interest is the observation that the cleaved channel protein is accumulated and retrieved from plasma membrane into the cell by endocytosis. Both proteolysis and recycling are



abolished by addition of C.I.2, a synthetic calpain inhibitor [25]. We have demonstrated that high amounts of cleaved CFTR form, revealed by the presence of the 100 kD fragment, are accumulated in growing JA3 cells and in human circulating lymphocytes, supporting the concept that this limited proteolysis of CFTR could represent the initial regulatory step of the normal turnover of the protein. In this respect, we have observed a linear correlation between the calpain activation and the level of cleaved CFTR. In fact, by reducing intracellular calpain activity a large increase in the amount of native CFTR at the plasma membrane was recovered in parallel with the disappearance of the cleaved form.

We also demonstrate that calpain activation, required for the limited degradation of CFTR, is induced by the NMDA receptor engagement. In fact, following exposure of growing JA3 cells to the NMDA receptor blocker MK801, the level of native CFTR at plasma membrane is increased in concomitance with the disappearance of the 100 kD fragment. These observations, together with the identification of a cleaved channel form in human circulating lymphocytes, clearly indicate that CFTR proteolysis by calpain is a naturally occurring process. We can speculate that, the alteration in the secretory function occurring in lymphocytes from CF patients [46, 47], could be triggered by an improper opening of the NMDA receptor and by the subsequent calpain activation. In this respect, it is interesting to note that some of the compounds, belonging to the family of benzoquinolinizinium compounds, suggested to cause rescuing of mutated CFTR at the plasma membrane, are also inhibiting  $\text{Ca}^{2+}$  influx through NMDA receptor [48-49].

We can also speculate that, cleavage of CFTR by calpain can promote a reorganization of the protein channel, inducing alterations in its PDZ domain binding capacity and internalization of the modified channel [5, 6]. Moreover, the higher affinity of digested CFTR for HSP90 can be considered an additional indication of the conformational changes induced by calpain on the channel protein. These modifications could be of particular importance at a functional level, since the stabilization of the cleaved CFTR form by HSP90 may favour its internalization process. On the basis of all these observations we can postulate that the protease sensitive region of CFTR may represent an important signal involved in the control of the functional channel level at plasma membrane. Once this signal sequence becomes accessible to an active protease, the cleaved CFTR is rapidly and irreversibly removed and addressed to further proteolytic processes. This selective limited proteolysis operated by calpain can be considered as a mechanism not restricted to CFTR since the involvement of the protease has been suggested to occur in the degradation of other ion channel proteins [19, 20, 50]. Thus, our findings are proposing a physiological function of the protease in the normal turnover of transmembrane proteins, including CFTR.

The present findings are summarized in the model presented in Fig. 10. Accordingly, we suggest that CFTR degradation by calpain at the plasma membranes is part of a mechanism responsible for the normal turnover of the channel protein in which a regulatory role is exerted by the chaperone HSP90. Since these processes occur in lymphocytes, these cells may be useful to monitor the effectiveness of proposed therapeutic treatments in CF patients.

### Acknowledgments

This work was supported in part by grants from Ministero dell'Istruzione dell'Università e della Ricerca (PRIN 2008), and from the University of Genoa.

### References

1. Prince, L.S., Workman, R.B. Jr., and Marchase, R.B. (1994) Rapid endocytosis of the cystic fibrosis transmembrane conductance regulator chloride channel. *Proc Natl Acad Sci U S A* **91**, 5192-5196.
2. Kleizen, B., Braakman, I., and de Jonge, H.R. (2000) Regulated trafficking of the CFTR chloride channel. *Eur. J. Cell. Biol.* **79**, 544-556. Review.
3. Moyer, B.D., Duhaime, M., Shaw, C., Denton, J., Reynolds, D., Karlson, K.H., Pfeiffer, J., Wang, S., Mickle, J.E., Milewski, M., Cutting, G.R., Guggino, W.B., Li, M., and Stanton, B.A.

- (2000) The PDZ-interacting domain of cystic fibrosis transmembrane conductance regulator is required for functional expression in the apical plasma membrane. *J. Biol. Chem.* **275**, 27069-27074.
4. Guggino, W.B., and Stanton, B.A. (2006) New insights into cystic fibrosis: molecular switches that regulate CFTR. *Nat. Rev. Mol. Cell. Biol.* **7**, 426-436.
  5. Cushing, P.R., Fellows, A., Villone, D., Boissguérin, P., and Madden, D.R. (2008) The relative binding affinities of PDZ partners for CFTR: a biochemical basis for efficient endocytic recycling. *Biochemistry* **47**, 10084-10098.
  6. Swiatecka-Urban, A., Duhaime, M., Coutermarsh, B., Karlson, K.H., Collawn, J., Milewski, M., Cutting, G.R., Guggino, W.B., Langford, G., and Stanton, B.A. (2002) PDZ domain interaction controls the endocytic recycling of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* **277**, 40099-40105.
  7. Haggie, P.M., Stanton, B.A., and Verkman, A.S. (2003) Increased diffusional mobility of CFTR at the plasma membrane after deletion of its C-terminal PDZ binding motif. *J. Biol. Chem.* **279**, 5494-5500.
  8. Ward, C.L., Omura, S., and Kopito, R.R. (1995) Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* **83**, 121-127.
  9. Jensen, T.J., Loo, M.A., Pind, S., Williams, D.B., Goldberg, A.L., and Riordan, J.R. (1995) Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* **83**, 129-135.
  10. Stratford, F.L., Pereira, M.M., Becq, F., McPherson, M.A., and Dormer, R.L. (2003) Benzo(c)quinolizinium drugs inhibit degradation of Delta F508-CFTR cytoplasmic domain. *Biochem Biophys Res Commun.* **300**, 524-530.
  11. Goll, D.E., Thompson, V.F., Li, H., Wei, W., and Cong, J. (2003) The calpain system. *Physiol. Rev.* **83**, 731-801.
  12. Hood, J.L., Brooks, W.H., and Roszman, T.L. (2006) Subcellular mobility of the calpain/calpastatin network: an organelle transient. *BioEssays* **28**, 850-859.
  13. Ribeiro, C.M., Paradiso, A.M., Carew, M.A., Shears, S.B., and Boucher, R.C. (2005) Cystic Fibrosis Airway Epithelial Ca<sup>2+</sup>i Signaling: the mechanism for the larger agonist-mediated Ca<sup>2+</sup>i signals in human cystic fibrosis airway epithelia. *J. Biol. Chem.* **280**, 10202-10209.
  14. Norez, C., Antigny, F., Becq, F., and Vandebrouck, C. (2006) Maintaining Low Ca<sup>2+</sup> Level in the Endoplasmic Reticulum Restores Abnormal Endogenous F508del-CFTR Trafficking in Airway Epithelial Cells. *Traffic* **7**, 562-573.
  15. Antigny, F., Norez, C., Cantereau, A., Becq, F., and Vandebrouck, C. (2008) Abnormal spatial diffusion of Ca<sup>2+</sup> in F508del-CFTR airway epithelial cells. *Respir. Res.* **9**, 1-17.
  16. Hung, A.Y., and Sheng, M. (2002) PDZ domains: structural modules for protein complex assembly. *J. Biol. Chem.* **277**, 5699-5702.
  17. Brône, B., and Eggermont, J. (2005) PDZ proteins retain and regulate membrane transporters in polarized epithelial cell membranes. *Am. J. Physiol. Cell. Physiol.* **288**, 20-29.
  18. Jemth, P., and Gianni, S. (2007) PDZ domains: folding and binding. *Biochemistry* **46**, 8701-8708.
  19. Yuen, E.Y., Ren, Y., and Yan, Z. (2008) Postsynaptic density-95 (PSD-95) and calcineurin control the sensitivity of N-methyl-D-aspartate receptors to calpain cleavage in cortical neurons. *Mol. Pharmacol.* **74**, 360-370.
  20. Aversa, M., Stifanese, R., De Tullio, R., Beccaria, F., Salamino, F., Pontremoli, S., and Melloni, E. (2009) Calpain-mediated activation of NO synthase in human neuroblastoma SK-N-BE cells. *J. Neurochem.* **110**, 412-421.
  21. Melloni, E., Sparatore, B., Salamino, F., Michetti, M., and Pontremoli, S. (1982) Cytosolic calcium dependent proteinase of human erythrocytes: formation of an enzyme-natural inhibitor complex induced by Ca<sup>2+</sup> ions. *Biochem. Biophys. Res. Commun.* **106**, 731-740.

22. Salamino, F., Averna, M., Tedesco, I., De Tullio, R., Melloni, E., and Pontremoli, S. (1997) Modulation of rat brain calpastatin efficiency by post-translational modifications. *FEBS Lett.* **412**, 433-438.
23. Averna, M., Stifanese, R., De Tullio, R., Salamino, F., Bertuccio, M., Pontremoli, S., and Melloni, E. (2007) Proteolytic degradation of nitric oxide synthase isoforms by calpain is modulated by the expression levels of HSP90. *FEBS J.* **274**, 6116-6127.
24. Stifanese, R., Averna, M., De Tullio, R., Salamino, F., Cantoni, C., Mingari, M.C., Prato, C., Pontremoli, S., and Melloni, E. (2008) Role of the calpain-calpastatin system in the density-dependent growth arrest. *Arch. Biochem. Biophys.* **479**, 145-152.
25. De Tullio, R., Stifanese, R., Salamino, F., Pontremoli, S., and Melloni, E. (2003) Characterization of a new p94-like calpain form in human lymphocytes. *Biochem. J.* **375**, 689-696.
26. Laemmli, U.K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **227**, 680 – 685.
27. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U S A.* **76**, 4350-4354.
28. Palejwala, S., and Goldsmith, L.T. (1992) Ovarian expression of cellular Ki-ras p21 varies with physiological status. *Proc. Natl. Acad. Sci.* **89**, 4202–4206.
29. DeTullio, R., Passalacqua, M., Averna, M., Salamino, F., Melloni, E., and Pontremoli, S. (1999) Changes in intracellular localization of calpastatin during calpain activation. *Biochem. J.* **343**, 467-472.
30. Averna, M., Stifanese, R., De Tullio, R., Passalacqua, M., Defranchi, E., Salamino, F., Melloni, E., and Pontremoli, S. (2007) Regulation of calpain activity in rat brain with altered  $\text{Ca}^{2+}$  homeostasis. *J. Biol. Chem.* **282**, 2656-2665.
31. Aronson, N.N., and Touster, O. (1974) Isolation of rat liver plasma membrane fragments in isotonic sucrose. *Methods Enzimol.* **31**, 90-102.
32. Amaral, M.D. (2004) CFTR and chaperones: processing and degradation. *J. Mol. Neurosci.* **23**, 41-48.
33. Du, K., Sharma, M., and Lukacs, G.L. (2005) The DeltaF508 cystic fibrosis mutation impairs domain-domain interactions and arrests post-translational folding of CFTR. *Nat. Struct. Mol. Biol.* **12**, 17-25.
34. Cui, L., Aleksandrov, L., Chang, X.B., Hou, Y.X., He, L., Hegedus, T., Gentzsch, M., Aleksandrov, A., Balch, W.E., and Riordan, J.R. (2007) Domain interdependence in the biosynthetic assembly of CFTR. *J. Mol. Biol.* **365**, 981-994.
35. Cull-Candy, S., Brickley, S., and Farrant, M. (2001) NMDA receptor subunits: diversity, development and disease. *Curr. Opin. Neurobiol.* **11**, 327-335.
36. Baravalle, G., Schober, D., Huber, M., Bayer, N., Murphy, R.F., and Fuchs, R. (2005) Transferrin recycling and dextran transport to lysosomes is differentially affected by bafilomycin, nocodazole and low temperature. *Cell. Tissue Res.* **320**, 99-113.
37. Xiong, X., Chong, E., and Skach, W.R. (1999) Evidence that endoplasmic reticulum (ER)-associated degradation of cystic fibrosis transmembrane conductance regulator is linked to retrograde translocation from the ER membrane. *J Biol Chem.* **274**, 2616-2624.
38. Sun, F., Mi, Z., Condliffe, S.B., Bertrand, C.A., Gong, X., Lu, X., Zhang, R., Latoche, J.D., Pilewski, J.M., Robbins, P.D., and Frizzell, R.A. (2008) Chaperone displacement from mutant cystic fibrosis transmembrane conductance regulator restores its function in human airway epithelia. *FASEB J.* **22**, 3255-3263.
39. Yetik-Anacak, G., Xia, T., Dimitropoulou, C., Venema, R. C., and Catravas, J. D. (2006) Effects of hsp90 binding inhibitors on sGC-mediated vascular relaxation. *Am. J. Physiol. Heart Circ. Physiol.* **291**, 260-268.

40. Wu, W., Kao, Y., Hu, P., and Chen, J. (2007) Geldanamycin, a HSP90 inhibitor, attenuates the hypoxia-induced vascular endothelial growth factor expression in retinal pigment epithelium cells in vitro. *Exp. Eye Res.* **85**, 721-731.
41. Ciechanover, A. (1994) The ubiquitin-proteasome proteolytic pathway. *Cell* **79**, 13-21.
42. Skach, W.R. (2000) Defects in processing and trafficking of the cystic fibrosis transmembrane conductance regulator. *Kidney Int.* **57**, 825-31. Review.
43. Milewski, M.I., Mickle, J.E., Forrest, J.K., Stafford, D.M., Moyer, B.D., Cheng, J., Guggino, W.B., Stanton, B.A., and Cutting, G.R. (2001) A PDZ-binding motif is essential but not sufficient to localize the C terminus of CFTR to the apical membrane. *J. Cell. Sci.* **114**, 719-726.
44. Kleizen, B., Braakman, I., and de Jonge HR. (2000) Regulated trafficking of the CFTR chloride channel. *Eur. J. Cell. Biol.* **79**, 544-556. Review.
45. Sarkadi, B., Bauzon, D., Huckle, W.R., Earp, H.S., Berry, A., Suchindran, H., Price, E.M., Olsen, J.C., Boucher, R.C., and Scarborough, G.A. (1992) Biochemical characterization of the cystic fibrosis transmembrane conductance regulator in normal and cystic fibrosis epithelial cells. *J. Biol. Chem.* **267**, 2087-2095.
46. Moss, R.B., Bocian, R.C., Hsu, Y.P., Dong, Y.J., Kemna, M., Wei, T., and Gardner, P. (1996) Reduced IL-10 secretion by CD4+ T lymphocytes expressing mutant cystic fibrosis transmembrane conductance regulator (CFTR). *Clin. Exp. Immunol.* **106**, 374-388.
47. Moss, R.B., Hsu, Y.P., and Olds, L. (2000) Cytokine dysregulation in activated cystic fibrosis (CF) peripheral lymphocytes. *Clin. Exp. Immunol.* **120**, 518-525.
48. Earley, W.G., Kumar, V., Mallamo, J.P., Subramanyam, C., Dority, J.A. Jr, Miller, M.S., DeHaven-Hudkins, D.L., Aimone, L.D., Kelly, M.D., and Ault, B. (1995) Novel benzo[b]quinolizinium cations as uncompetitive N-methyl-D-aspartic acid (NMDA) antagonists: the relationship between log D and agonist independent (closed) NMDA channel block. *J. Med. Chem.* **38**, 3586-3592.
49. Dormer, R.L., Dérand, R., McNeilly, C.M., Mettey, Y., Bulteau-Pignoux, L., Métayé, T., Vierfond, J.M., Gray, M.A., Galletta, L.J., Morris, M.R., Pereira, M.M., Doull, I.J., Becq, F., and McPherson, M.A. (2001) Correction of  $\Delta$ F508-CFTR activity with benzo(c)quinolizinium compounds through facilitation of its processing in cystic fibrosis airway cells. *J. Cell. Sci.* **114**, 4073-4081.
50. von Reyn, C.R., Spaethling, J.M., Mesfin, M.N., Ma, M., Neumar, R.W., Smith, D.H., Siman, R., and Meaney, D.F. (2009) Calpain mediates proteolysis of the voltage-gated sodium channel  $\alpha$ -subunit. *J. Neurosci.* **29**, 10350-10356.

### Legend to figures

#### Figure 1. CFTR forms in FRT, JA3 cells and human circulating lymphocytes.

(A) Aliquots (5  $\mu$ g) of total membrane fraction prepared from FRT cells were incubated at 37°C in SDS-PAGE loading buffer for 30 min and submitted to 6 % SDS-PAGE, followed by immunoblotting. CFTR was detected using clone M3A7 (lane 1) or clone 24-1 (lane 2) as primary antibodies. Aliquots (20  $\mu$ g) of total membrane fraction prepared from JA3 cells (B) and human circulating lymphocytes (C) incubated at 37°C in the same conditions described in (A) were submitted to 6 % SDS-PAGE followed by immunoblotting. CFTR was detected using clone M3A7 (lane 1) or clone 24-1 (lane 2) as primary antibodies.

The immunoreactive bands of each lane 1 were quantified as described in Experimental. The values reported are the arithmetic mean  $\pm$  S.D. of three different experiments

#### Figure 2. *In vitro* digestion of CFTR from FRT and JA3 cells by calpain-mediated limited proteolysis.

(A) Aliquots (5  $\mu$ g) of total membrane fraction, prepared from FRT cells expressing wild-type CFTR, were incubated at 37°C with 1 unit of calpain. At the indicated times, digestions were stopped by addition of Laemmli SDS-PAGE loading solution. The mixtures were incubated at 37°C for 30 min and submitted to 6% SDS-PAGE, followed by immunoblotting. CFTR was detected using the specific antibody (clone M3A7). Alternatively, the total membrane fraction was also



subjected to limited proteolysis by using 0.2  $\mu\text{g}$  of trypsin on ice (Try). **(B)** Aliquots (20  $\mu\text{g}$ ) of total membrane fraction prepared from JA3 cells were incubated at 37°C with 1 unit of calpain in the same conditions described in (A).

The immunoreactive bands, detected in the upper panels of (A) and (B), were quantified as described in Methods. The values, reported in the lower panels, are the arithmetic mean  $\pm$  S.D. of three different experiments, corrected on the basis of the amount of protein present in each lane.

**(C)** Aliquots (10  $\mu\text{g}$ ) of native (N.) and digested (D.) CFTR prepared from FRT cells, as described above, were washed in EDTA 1mM and then solubilized in buffer C, containing 0.5 M KCl and 0.1% Triton<sup>®</sup> X-100 for 2 hours at 4°C (see Methods). The samples were loaded on to a 0.8% agarose gel, followed by immunoblotting as described in Methods. CFTR was detected using the specific antibody (clone M3A7).

**Figure 3. Changes in the level and localization of CFTR in FRT cells loaded with  $\text{Ca}^{2+}$ .**

**(A)** FRT cells ( $2 \times 10^6$ ) were treated for 1 hour at 37°C with 1  $\mu\text{M}$   $\text{Ca}^{2+}$ -ionophore A23187 in the absence ( $\text{Ca}^{2+}$ ) or the presence of 1  $\mu\text{M}$  C.I.2 ( $\text{Ca}^{2+}$  + C.I.2). Cells were then lysed and aliquots (20  $\mu\text{l}$ ) of total membrane fraction were solubilized in Laemmli SDS-PAGE loading solution and submitted to SDS-PAGE 6% followed by immunoblotting. CFTR was detected with mAb M3A7.

**(B)** CFTR protein bands, derived from the experiments described in (A), were quantified as described in Experimental. The values are reported as the arithmetic mean  $\pm$  S.D. of three different experiments, corrected on the basis of the amount of protein present in each lane.

**(C)** FRT cells ( $10 \times 10^4$  cells), grown on glass slides, were treated for 30 min with 1  $\mu\text{M}$   $\text{Ca}^{2+}$ -ionophore A23187 in the absence (+  $\text{Ca}^{2+}$ ) or the presence of 1  $\mu\text{M}$  C.I.2 (+  $\text{Ca}^{2+}$  + C.I.2). After treatment, CFTR localization was determined by confocal microscopy using mAb M3A7. The images are representative of six different experiments. The arrows indicate CFTR associated to plasma membrane and to cytosolic spots. The CFTR fluorescence was quantified as described in Experimental. The values reported are the arithmetic mean  $\pm$  S.D. of six different experiments.

**Figure 4. Correlations between intracellular calpain activity and levels of the 100 kD CFTR segment.**

**(A). Changes in CFTR levels in JA3 cells loaded with  $\text{Ca}^{2+}$ .**

JA3 cells ( $2 \times 10^6$ ) were treated for 1 hour at 37°C with 1  $\mu\text{M}$   $\text{Ca}^{2+}$ -ionophore A23187 in the absence ( $\text{Ca}^{2+}$ ) or the presence of 1  $\mu\text{M}$  C.I.2 ( $\text{Ca}^{2+}$  + C.I.2). Cells were then lysed and aliquots (40  $\mu\text{l}$ ) of total membrane fraction were solubilized in Laemmli SDS-PAGE loading solution and submitted to SDS-PAGE 6%. Following immunoblotting, CFTR protein bands, detected with mAb M3A7, were quantified as described in Experimental. The values, reported in the lower panel, are the arithmetic mean  $\pm$  S.D. of three different experiments, corrected on the basis of the amount of protein present in each lane.

**(B). Digestion of CFTR in growing JA3 cells.**

JA3 and JA3 overexpressing cast48 (JA3-cast) cells were incubated at a density of  $5 \times 10^4/\text{ml}$  as described in Experimental. JA3 cells were grown for 24 hours in the absence (JA3) or in the presence of 1  $\mu\text{M}$  C.I.2 (JA3 + C.I.2). JA3 + C.I.2 cells were then treated with 1  $\mu\text{M}$   $\text{Ca}^{2+}$ -ionophore A23187 for 1 hour (JA3 + C.I.2 +  $\text{Ca}^{2+}$ ). Alternatively, JA3-cast cells were treated with 1  $\mu\text{M}$   $\text{Ca}^{2+}$ -ionophore A23187 for 1 hour (JA3-cast +  $\text{Ca}^{2+}$ ). Aliquots corresponding to  $50 \times 10^4$  cells were lysed 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 the specific antibody. The immunoreactive bands corresponding to 170 kD (white bars) and 100 kD (light grey bars), derived from the experiments described in the upper panel, were quantified (see Experimental). Aliquots corresponding to  $2 \times 10^5$  cells of the experiments, described in the upper panel, were treated as previously described [24] to measure intracellular calpain activity at 30 min following the addition of 0.1  $\mu\text{M}$  calcium ionophore A23187 (grey bars). The values reported are the arithmetic mean  $\pm$  S.D. of three different experiments.

**Figure 5. Intracellular localization of CFTR corresponding to 170 and 100 kD bands.**

Total membrane fraction (0.5 ml) prepared from JA3 (A) or JA3-cast cells (B), as described in Experimental, was fractionated on a discontinuous sucrose density gradient (see Experimental). Aliquots (30  $\mu$ l) of plasma and intracellular membranes from JA3 cells 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. 5'-Nucleotidase (5'-NT) activity was assayed as described in [31].

**Figure 6. Susceptibility to calpain digestion of mature and immature CFTR.**

(A) Aliquots (10  $\mu$ g) of total membrane fraction, prepared from FRT cells expressing wild-type CFTR, were incubated at 37°C with 1 unit of calpain in the absence or presence of 2  $\mu$ g of HSP90. After 10 min, digestions were stopped by addition of Laemmli SDS-PAGE loading solution. The mixtures were incubated at 37°C for 30 min and submitted to 6% SDS-PAGE, followed by immunoblotting. CFTR was detected using the specific antibody (clone M3A7). (B) FRT cells ( $10 \times 10^6$ ) were treated at 37°C with 1  $\mu$ M  $\text{Ca}^{2+}$ -ionophore A23187 ( $\text{Ca}^{2+}$ ) for the indicated times. Cells were then lysed and aliquots (20  $\mu$ l) of total membrane fraction were solubilized in Laemmli SDS-PAGE loading solution and submitted to SDS-PAGE 6% followed by immunoblotting. CFTR was detected with mAb M3A7.

**Figure 7. Effect of MK801 and bafilomycin on CFTR levels in growing JA3 cells.**

JA3 cells were incubated at a density of  $5 \times 10^4$ /ml as described in Experimental. JA3 cells were grown for 24 hours in the absence (control, white bars) or in the presence of 1  $\mu$ M MK801 (MK, light grey bars) or in the presence of 10 nM bafilomycin (BAF, grey bars). Aliquots corresponding to  $50 \times 10^4$  cells were lysed 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 the specific antibody. The immunoreactive bands corresponding to 170 kD and 100 kD were quantified (see Experimental) and the values reported are the arithmetic mean  $\pm$  S.D. of three different experiments.

**Figure 8. CFTR susceptibility to calpain digestion following its interaction with HSP90.**

(A) Aliquots (5  $\mu$ g) of the cell membranes containing CFTR corresponding to 170 kD and 100 kD bands from FRT cells, prepared as described in Experimental, were solubilized in 0.1% Triton<sup>®</sup> X-100. Aliquots (5  $\mu$ g) of HSP90 were added to the membrane suspension and used for immunoprecipitation (I.P.). The immunocomplexes were detected by immunoblotting (WB) using the indicated antibodies.

(B) and (C) Aliquots (2  $\mu$ g) of the same cell membranes used in (A) and containing CFTR corresponding to 170 kD and 100 kD bands were incubated at 37°C with 1 unit of calpain in the absence or presence of 2  $\mu$ g of HSP90, or alternatively in the presence of 2  $\mu$ g of BSA. At the indicated times, the samples were suspended 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. The immunoreactive bands were quantified as described in Experimental. The values, normalized on the basis of the amount of protein present in each lane, are reported as the arithmetic mean  $\pm$  S.D. of three different experiments.

**Figure 9. Effect of geldanamycin on CFTR levels in FRT cells.**

FRT cells ( $10 \times 10^4$  cells) grown on glass slides were treated for 30 min with 100 nM GA alone (GA) or with the addition of 1  $\mu$ M  $\text{Ca}^{2+}$ -ionophore A23187 in the absence ( $\text{Ca}^{2+}$  + GA) or the presence of 1  $\mu$ M C.I.2 ( $\text{Ca}^{2+}$  + GA + C.I.2). After treatment, CFTR localization was determined by confocal microscopy using mAb M3A7. The CFTR fluorescence was quantified as described in Experimental. The values reported are the arithmetic mean  $\pm$  S.D. of six different experiments.

**Figure 10. Proposed model for CFTR turnover.**

Following a rise in  $[\text{Ca}^{2+}]_i$ , active calpain, translocated to the plasma membranes, cleaves native CFTR at a site probably located between the first nucleotide binding domain and the regulatory domain. The modified channel form is rapidly retrieved by endocytosis into the cell, where it is largely accumulated. The complete proteolysis of CFTR is then accomplished by the lysosomal pathway.

ER, endoplasmic reticulum membranes; PM, plasma membranes; VS, vesicle membranes.

Accepted Manuscript

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20100344

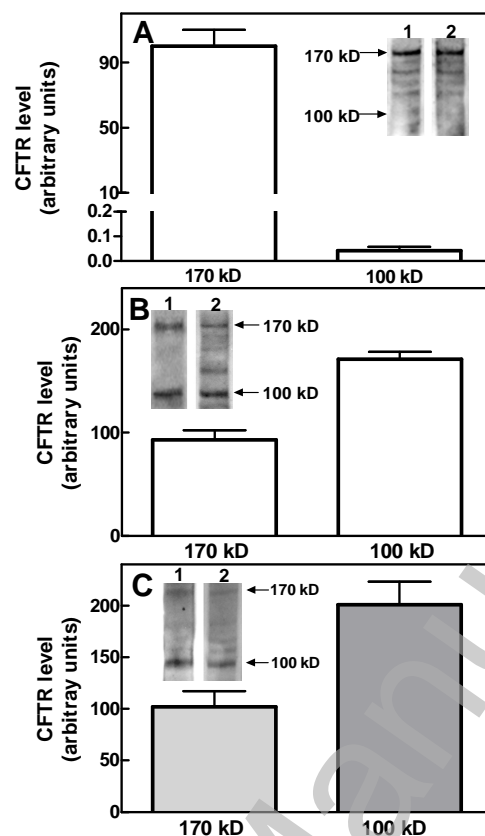


Figure 1



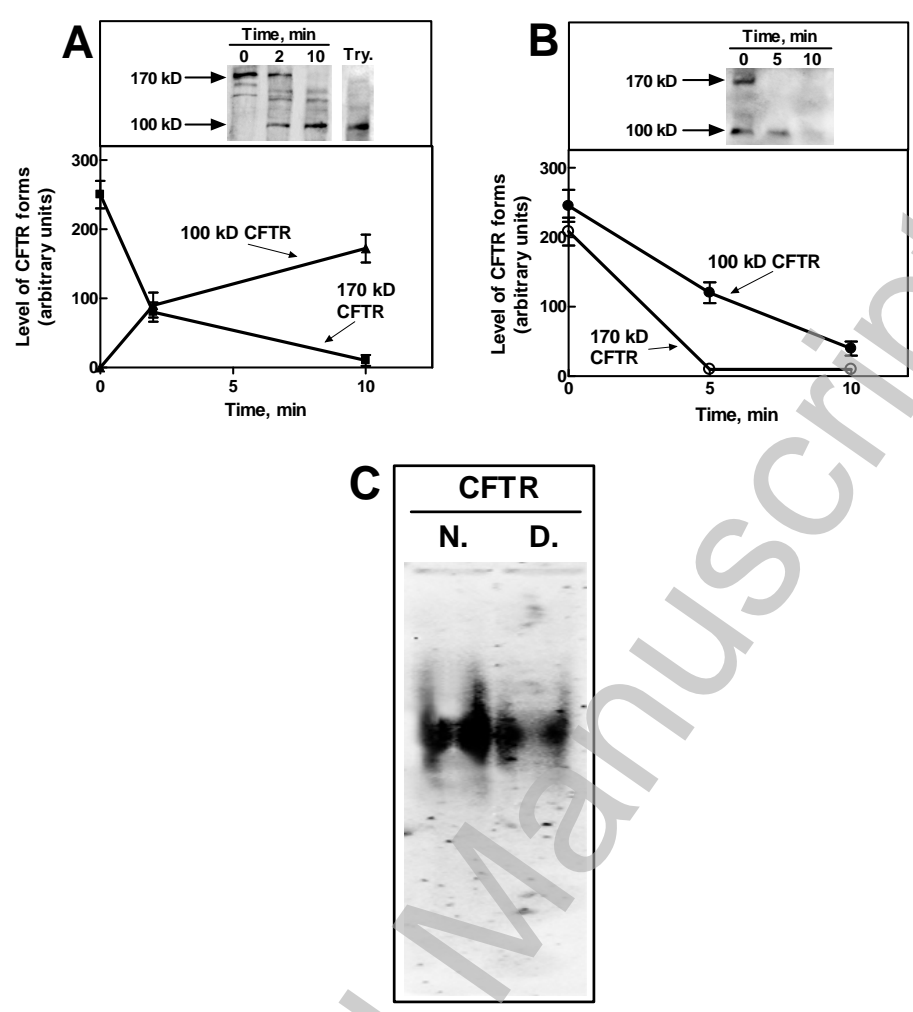


Figure 2

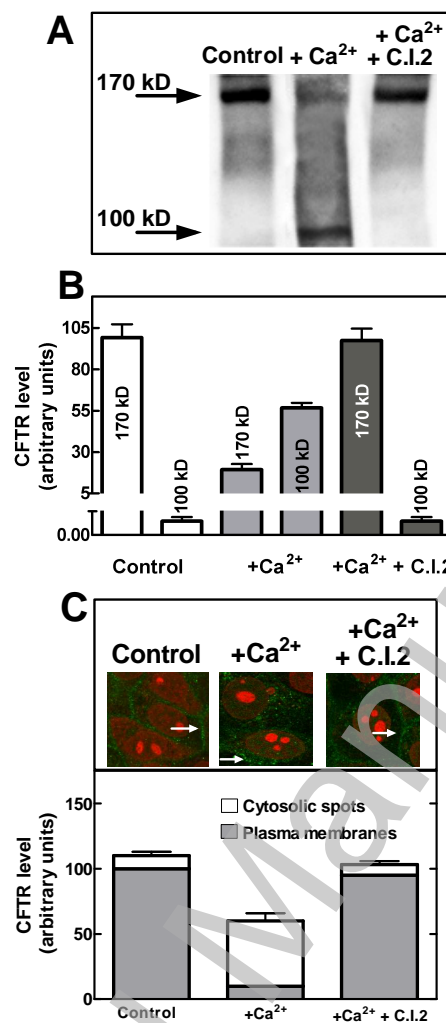
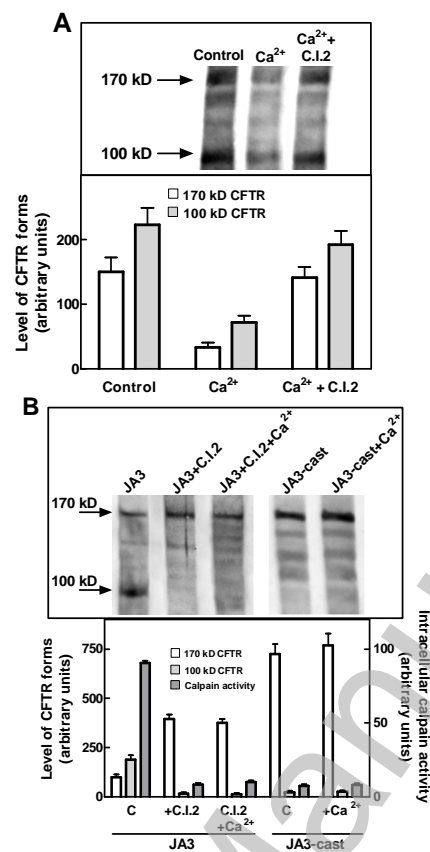


Figure 3



**Figure 4**

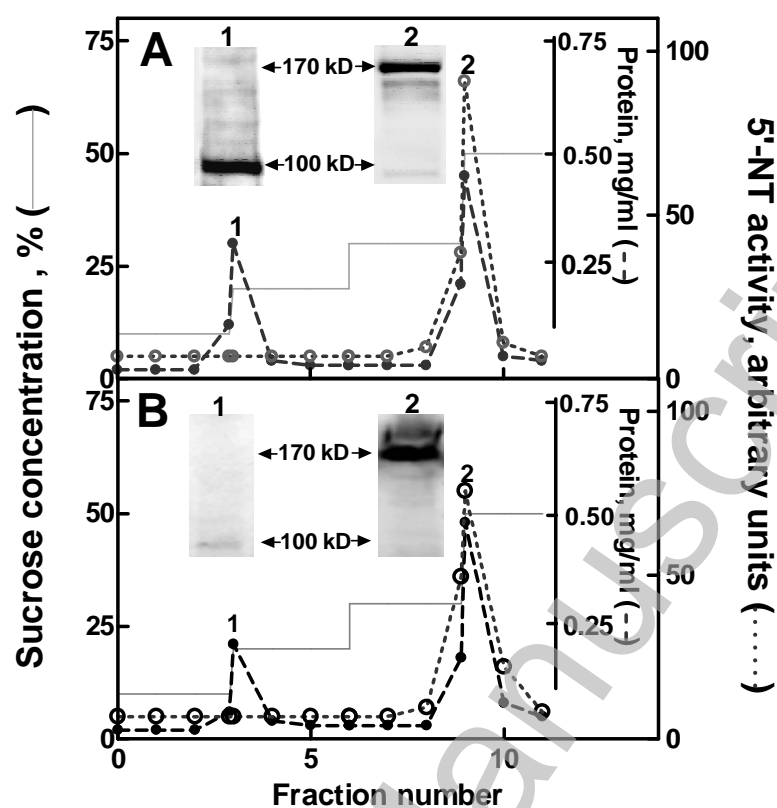
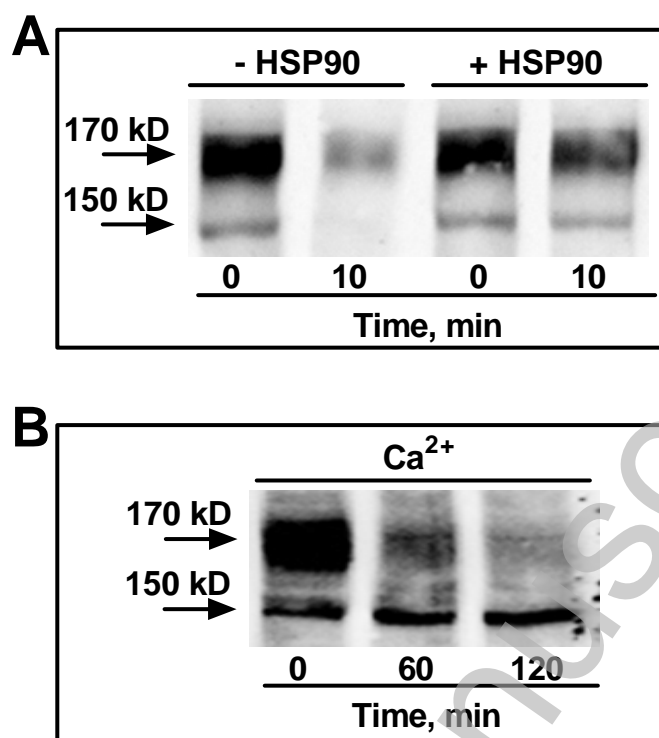


Figure 5





**Figure 6**

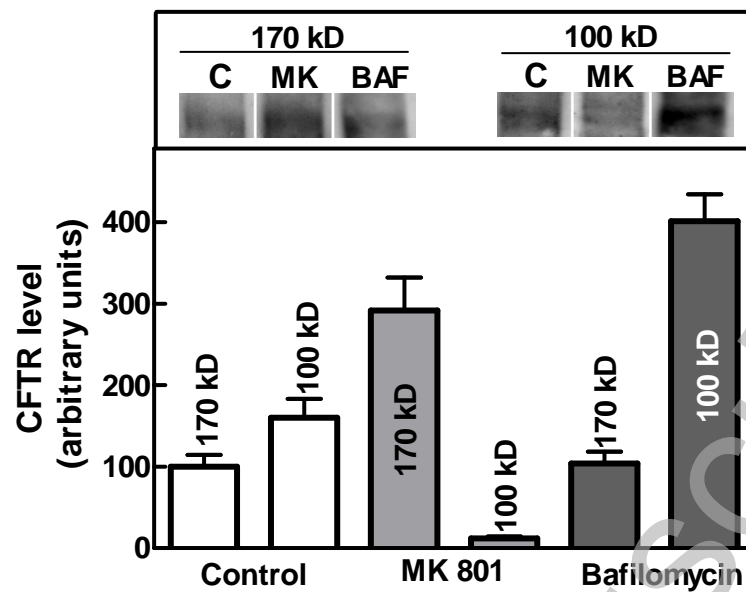


Figure 7

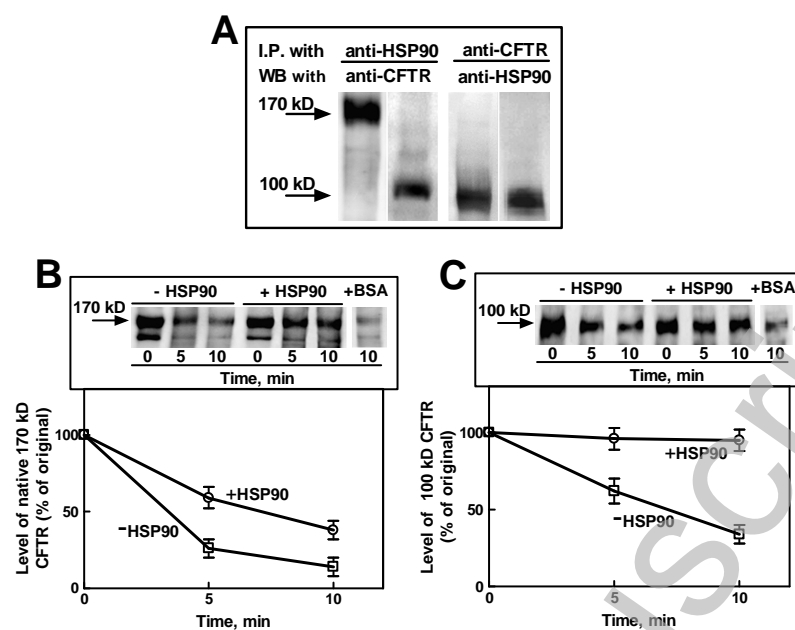


Figure 8

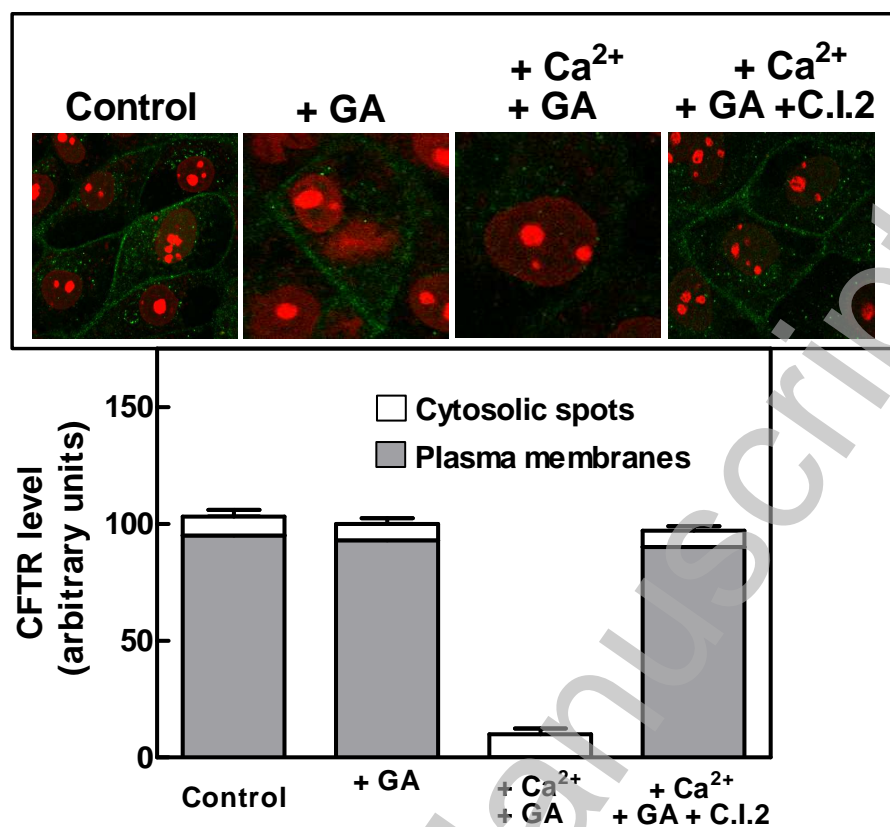


Figure 9



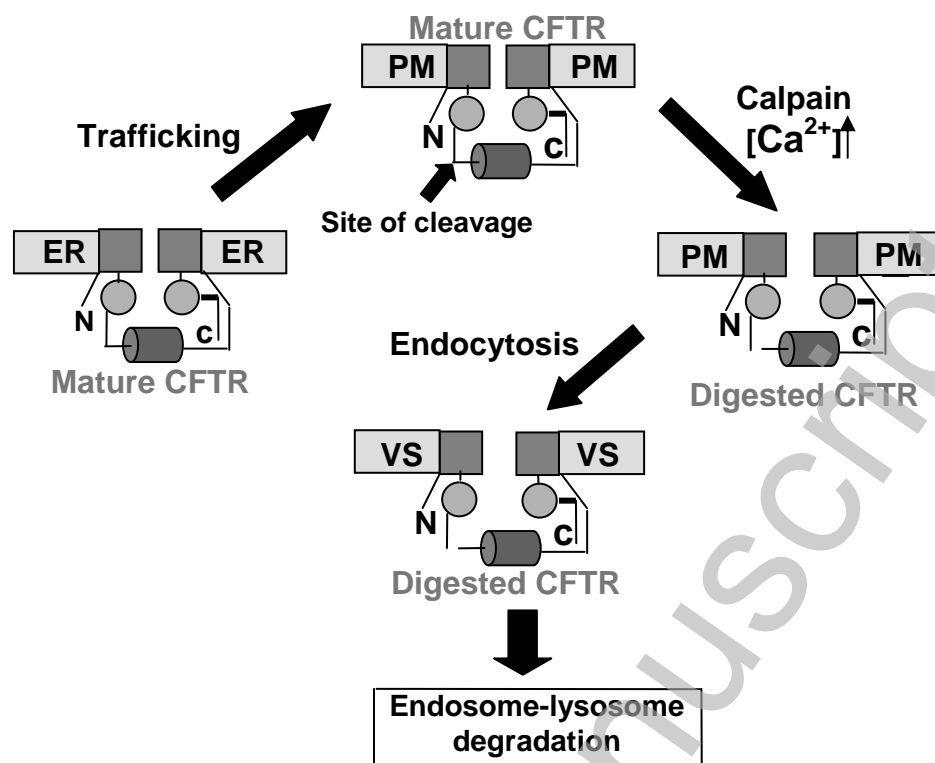


Figure 10