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**Annexin V is directly involved in cystic fibrosis transmembrane conductance regulator's
chloride channel function**

Short title: Annexin V - CFTR direct interaction

Pascal Trouvé, Marie-Anne Le Drévo, Mathieu Kerbiriou, Gaëlle Friocourt, Yann Fichou,
Danièle Gillet, Claude Férec.

INSERM, Unité 613, 46 rue Félix le Dantec, BP62025, 29220 Brest, France.

Tel: 33.(0)2.98.01.81.47

Fax: 33.(0)2.98.01.83.42

Corresponding authors: Claude Férec (claude.ferec@univ-brest.fr) and Pascal Trouvé
(pascal.trouve@univ-brest.fr).

SUMMARY

The cystic fibrosis transmembrane conductance regulator (CFTR) functions as a cAMP-activated chloride channel, which is regulated by protein-protein interactions. The extent to which CFTR is regulated by these interactions remains unknown. Annexin V is overexpressed in cystic fibrosis (CF), and given the functional properties of annexin V and CFTR we considered whether they are associated and if so whether this has implications for CFTR function. Using co-immunoprecipitation and overlay experiments, we show that annexin V is associated with nucleotide-binding domain 1 (NBD1) of CFTR. Surface plasmon resonance (SPR) indicated different K_D values in the absence and presence of both calcium and ATP, suggesting that this interaction is calcium- and ATP-dependent. Using an siRNA approach and overexpression, we showed that CFTR chloride channel function and its localization in the cell membranes were dependent on annexin V expression. We concluded that annexin V is necessary for normal CFTR chloride channel activity. Furthermore, we show that CFTR and annexin V are partially co-distributed in normal epithelial cells in human bronchi. In conclusion, we show for the first time that annexin V is associated with CFTR and is involved in its function.

Keywords: protein-protein interaction, normal CFTR function, protein localization.

INTRODUCTION

Cystic fibrosis (CF) is a lethal autosomal recessive disease characterized by defects in epithelial ion transport [1]. CF is caused by mutations in both CFTR alleles encoding the cystic fibrosis transmembrane conductance regulator (CFTR) [1, 2]. This ATP-binding cassette transporter functions as a chloride (Cl⁻) channel [2, 3, 4] and comprises two hydrophobic core regions, two nucleotide-binding domains (NBDs) with ATP-binding activity [5] and a regulatory domain (R domain). CFTR channel opening requires phosphorylation by cAMP-dependent protein kinases (PKA, 6) and hydrolyzable MgATP [7, 8].

Since the CFTR gene was cloned in 1989, more than 1300 mutations have been described and, despite being a monogenic disease, CF appears to be very heterogeneous with a phenotype that ranges from nonclinical to clinical (congenital absence of vas deferens, mild pulmonary disease and idiopathic chronic pancreatitis). The severity of CF phenotype is influenced by other factors including gene modifiers and interactions between CFTR and its partners. Although several attempts have been made to correlate phenotype and genotype among patients sharing the same mutations, this correlation remains elusive and both genetic and environmental factors seem to be involved.

CFTR regulation is complex and involves dimerization of the protein [9, 10] and interdomain interactions [11]. Syntaxin 1A, EBP50, E3KARP, the μ subunit of the endocytic clathrin adaptor complex and cysteine string proteins are CFTR-binding proteins [12, 13, 14, 15, 2], but the extent to which CFTR channels are regulated by protein-protein interactions remains largely unknown.

Our aim was to identify new wild-type CFTR-binding proteins to improve our understanding of the pathophysiology of CF. We focused on annexin V which is overexpressed in CF epithelial cells from foetal trachea [16]. Although the exact role of annexin V in cells is

unknown, it has been shown to bind to phosphatidylserine (PS) in the presence of calcium [17, 18, 19]. This property is shared by the NBD1 domain of CFTR [20], indicating that the two proteins may have the same cellular location. We therefore hypothesized that annexin V and CFTR may be co-distributed in cells and maybe interact. We also hypothesized that there is a possible functional link between the two proteins, since annexin V was recently shown to interact with membrane proteins and to regulate the sodium-calcium exchanger and caveolin in cardiomyocytes [21].

Using co-immunoprecipitation, overlay experiments and surface plasmon resonance (SPR), we show that annexin V and CFTR interact directly. Furthermore, we show that this interaction involves the NBD1 domain of CFTR. In addition, this interaction is reinforced in the presence of both calcium ions and MgATP, as shown by Scatchard analysis of the dissociation constants (K_D) of the interaction in the absence and presence of calcium and ATP. The chloride channel function of CFTR was studied in A549 cells in the presence of siRNA directed against annexin V and when annexin V was overexpressed. Functional experiments (SPQ) showed that the decreased expression of annexin V was correlated with a decreased CFTR chloride channel function due to a decreased CFTR accumulation in the cell membranes. Conversely, overexpression of annexin V increased CFTR function and membrane localization. Therefore, we have shown for the first time that annexin V interacts directly with CFTR and that normal CFTR chloride function requires the presence of annexin V in cells. Confocal microscopy showed that annexin V and CFTR are partially co-distributed in human bronchial cells, indicating the physiological relevance of our findings.

METHODS

Protein extraction and co-immunoprecipitation

Normal human tissues from the upper airways (nasal tissues) were obtained from the Hôpital de la Cavale Blanche (Brest, France) following approval by the local ethics committee. The tissues were homogenized in lysis buffer (50 mM Tris HCl, 100 mM NaCl, 1% Triton X-100, 1.1 μ M leupeptin, 0.7 μ M aprotinin, 120 μ M PMSF, 1 μ M iodoacetamide, 0.7 μ M pepstatin and 1 mM DIFP; all from Sigma) using a polytron. Co-immunoprecipitations were carried out with Dynabeads (DynaL Biotech) coated with monoclonal anti-CFTR antibodies (10 μ g/ml, MM 13-4, RD System). The presence of CFTR protein in the complex was assessed and compared with a negative control in which the beads were coated with an irrelevant antibody directed against mouse Ig (not shown). A second control was performed by incubating the coated beads with the proteins extracted from untransfected A549 cells, which do not express the CFTR protein. The role of calcium was also checked by performing similar co-immunoprecipitation with CaCl_2 (10^{-5} M), EDTA or EGTA (10^{-3} M). After incubation, the beads were extensively washed and the samples were analyzed by Western blot (12% gel electrophoresis) using a polyclonal anti-annexin V antibody (1/1000, sc-1929 Invitrogen). Controls were performed without primary antibody, without secondary antibody (not shown).

Cell culture and transfection

A549 cells, an alveolar type II epithelium-like cell line provided by the American Type Culture Collection (Rockville, MD), were cultured in Ham's F-12 medium (M.E. Kaighn's modified medium) supplemented with 10% foetal calf serum, 50 mg/ml streptomycin and 50 U/ml penicillin in a 5% CO_2 -balanced air incubator at 37°C.

A549 cells were further transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions, with the full-length normal human CFTR cDNA (Transgene SA, pTG5985, France, access Genbank no. M28668) inserted in pcDNA3.1 *myc*-His (Invitrogen) between the KpnI and XhoI restriction sites, providing a C-terminal HisX6 tail. Stably transfected

clones were isolated by neomycin selection. After 10 passages, the expression of the CFTR protein was assessed by immunofluorescent labelling and Western blotting (not shown). Δ F508-CFTR expressing cells were also generated. Mutagenesis was performed on the cDNA encoding the CFTR protein, using the QuickChange® XL Site-directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, USA). The mutated oligonucleotides (Eurobio, France) that correspond to the Δ F508 mutation were: 5'-CTGGCACCATTAAAGAAAATATCGGTGTTTCCTATGATG-3'; 5'-CATCATAGGAAACACCGATATTTCTTTAATGGTGCCAG-3'. The Δ F508 mutation was controlled by double-strand sequencing (ABI 310, Applied Biosystem, USA) and Western blotting (not shown).

In A549 cells stably expressing CFTR, annexin V was inhibited using an siRNA specifically designed to knockdown gene expression according to the manufacturer's instructions (Santa Cruz Biotechnology). siRNA buffer alone, or an irrelevant scrambled siRNA provided by the manufacturer, was used as a negative control according to the manufacturer's instructions. According to the manufacturer (Santa Cruz Biotechnology), the scrambled sequence does not lead to the specific degradation of any cellular mRNA. Annexin V and actin expression was assessed by Western blotting, after 12% gel electrophoresis, using anti-annexin V (sc-1929) and anti-nonmuscle-F-actin (cytoskeleton, 1/1000 and 1/200, respectively, both from Santa Cruz Biotechnology), in the same protein extracts, at different periods of time after annexin V-silencing siRNA transfection. Densitometric analysis of the films was performed using a Biovision 1000 apparatus (Fischer Bioblock). Cell viability was assessed by the trypan blue exclusion test when siRNA was used (not shown).

In A549 cells stably expressing CFTR, annexin V overexpression was performed. The annexin V cDNA was amplified by PCR using complete cDNA from HeLa cells as the template. The primers used were sense 5'-ACCTGAGTAGTCGCCATGGCA-3' and anti-sense 5'-CCCCGTGACACGGTCATCTTC-3'. Reactions were carried out with the following parameters:

denaturation at 94°C for 15 min, annealing at 62°C for 30 min, and extension at 68°C for 1.5 min, for a total of 30 cycles. After gel electrophoresis, PCR products were purified with Qiaex II (Qiagen, Germany) and cloned into pcDNA3.1/His (TOPO TA Expression Kit, Invitrogen, USA) according to the manufacturer's instruction. Double-strand sequencing (ABI 310, Applied Biosystems, USA) were performed on recombinant clones to ascertain that PCR amplification did not introduce mistakes and the coding sequences were in frame. Annexin V overexpression in cells was assessed by Western blotting as described above at different post-transfection times.

Protein purification and overlay assay

The cDNA encoding NBD1 (nucleotides 420 to 650) was amplified by PCR using the human CFTR cDNA as template and cloned into a baculovirus vector encoding a C-terminal HisX6 tail (plasmid PVL1393, BD Biosciences). The cDNA encoding the NBD1 domain was 33 amino acid residues longer than the previously described human NBD1 domain, which was 453 to 650 [23]. The NBD1 peptide was produced in Sf9 insect cells (BaculoGold expression system, BD Biosciences) which were lysed in lysis buffer (50 mM Tris HCl, 100 mM NaCl, 1% Triton X-100 or 4% sodium pentadecafluorooctanoate (PFO, 24), 1.1 μ M leupeptin, 0.7 μ M aprotinin, 120 μ M PMSF, 1 μ M iodoacetamide, 0.7 μ M pepstatin and 1 mM DIFP; all from Sigma). Homogenates were subjected to 100,000 g centrifugation and NBD1-His was purified on an Ni-NTA column (Qiagen) as previously described [25]. Elution was performed with imidazole-containing buffer (pH 6.8), which has been shown to be the most efficient for this purpose [26].

Using the same methodology as for NBD1, the full-length CFTR and the Δ 508-CFTR protein were purified from the stably transfected A549 cells. Pure annexin V protein was from Sigma.

Before overlay assay, CFTR, NBD1 and annexin V were subjected to SDS/PAGE. In

order to assess the purity of the proteins, the gels were stained with NiAg. The purity of the CFTR, NBD1 and annexin V was further assessed by Western blotting. The antibodies for CFTR and NBD1 detection were MM13-4 (1/1000, RD) and L12B4 (1/1000, Neo Markers), respectively.

For overlay assays, annexin V (5 μg) was subjected to electrophoresis (12% polyacrylamide gel) and transferred to a PVDF membrane (Amersham, France), which was incubated with either purified NBD1 (10 $\mu\text{g/ml}$) or CFTR (10 $\mu\text{g/ml}$) in TBS buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween 20, pH 7.5) in the presence of 10^{-5} M CaCl_2 or 10^{-3} M EDTA, as previously described [27]. The membranes were then incubated with the anti-CFTR or the anti-NBD1 monoclonal antibody and the blots were developed after incubation with secondary antibodies using the ECLplus kit (Amersham). Control incubations omitting CFTR, omitting NBD1 or omitting the primary or secondary antibodies were performed. The specificity of the interaction was checked by using BSA as an irrelevant protein instead of annexin V.

Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed using a Biacore X apparatus. To test the interaction between annexin V and the full-length CFTR protein and between annexin V and the NBD1 domain, histidine-tagged CFTR or NBD1 proteins were immobilized by nickel chelation onto a NTA sensor chip, according to the manufacturer's instructions (Biacore). The NTA sensor chip, on which nitrilotriacetic acid is covalently linked to a carboxy-methylated dextran matrix, was activated by a pulse of NiCl_2 (500 μM), forming a chelating complex with NTA, which binds the polyhistidine peptides. The immobilization of the ligand (CFTR-His or NBD1-His) was adjusted to a low level (2000 RU), such that mass transport limitation and rebinding of the analyte to the immobilized ligand was minimal [28]. The injection

of an anti-CFTR antibody directed against the R-domain (MAB 1660, RD) and the injection of an anti-NBD1 antibody (L12B4, Neo Markers) were used in order to control the immobilized proteins (not shown). In a first set of experiments, different amounts of annexin V (0.50, 0.75 and 1 μ M) diluted in HBS-P (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% surfactant P20, Biacore) were injected (20 μ l/min) onto CFTR. The involvement of MgATP (0.5, 1, 1.5 and 2 mM) and Ca^{2+} (100, 500 and 1000 nM using CaCl_2) in the interaction between annexin V and NBD1 was studied. The binding of annexin V was evaluated 10, 30 and 100 seconds into the dissociation phase. An irrelevant protein (BSA) was used as negative control. For each experiment, the final curves were obtained by subtraction of the signal corresponding to the empty flow cell, which was Ni charged (flow cell 1).

In the case of the NBD1-annexin V interaction, quantitative measurements were performed in the absence and presence of physiological Ca^{2+} concentration (500 nM) and MgATP. For each concentration of annexin V, steady-state binding (R_{eq}) was measured and used to calculate the equilibrium constant K_D . The following equation was used, according to the BIA applications handbook (Biacore):

$$R_{\text{eq}} / C = K_A \times R_{\text{max}} - K_A \times R_{\text{eq}}$$

where C is the annexin V concentration, R_{max} is the total surface binding capacity in RU and R_{eq} is the steady-state binding in RU obtained when the equilibrium was reached for each concentration of annexin V. A plot of R_{eq} / C against R_{eq} at different concentrations gave a Scatchard plot from which K_D was calculated.

SPR was also used to assess the specificity of the NBD1-annexin V interaction. 8-cyclopentyl-1,3-dipropylxanthine (CPX, Sigma) which binds to the wild-type and to the mutated NBD1 was injected on the immobilized wild-type and on the delF508-CFTR proteins in order to

saturate the NBD1 sites. Annexin V was further injected to assess the inhibition of the interaction when the NBD1 sites were blocked. To assess whether the annexin V binding to NBD1 might be impeded by the delFF508 mutation, the mutated protein was immobilized and annexin V was injected. Negative controls were performed using an irrelevant protein (BSA) instead of CPX or instead of annexin V (not shown).

Each SPR experiment was performed at least three times.

SPQ fluorescence assay

Several cell lines were used in the 6-methoxy-N-(3-sulphopropyl) quinolinium (SPQ) experiments: 1) A549 cells, 2) A549 cells stably expressing CFTR 3) A549 cells transfected with both the CFTR and the annexin V cDNAs, and 4) A549 cells transfected with both the CFTR cDNA and the siRNA directed against annexin V, in 6-well plates. Fluorescence assays were performed as previously described [29] using forskolin as a CFTR activator. Cells were loaded with 10 mM SPQ and placed in a quenching NaI buffer. The baseline fluorescence was measured in isotonic NaI buffer and cells were then perfused with isotonic dequench buffer (NaNO₃ replaced NaI). The perfusate was then switched to dequench buffer plus agonist and requenched at the end of the experiments. Each SPQ assay was performed at least five times with different cell cultures. During the SPQ experiments the cell morphology and the cell number per microscopic field were checked in order to ensure that there was no difference between the cells in the presence or absence of siRNA.

SPQ experiments were also performed when annexin V was overexpressed in cells that were transfected or not with the cDNA encoding CFTR. The methodology was the same as described above. The controls were performed using cells expressing or not expressing CFTR which were transfected with the empty pcDNA3.1 vector.

In vitro phosphorylation of CFTR by PKA

Phosphorylation of the purified wild-type CFTR was carried out over the range 0 – 800 Units/ml PKA (cAMP-dependent protein kinase, catalytic subunit, Promega, France) using a luminescent kinase assay (Kinase-Glo-Plus Luminescent Kinase Assay, Promega). This luminescent assay provides a homogeneous method of determining any kinase activity based on the quantitation of ATP. The generated luminescent signal is proportional to the amount of ATP present in the reaction and inversely correlated with the amount of kinase activity. Experiments were performed in multiwell plates, in triplicate, using a previously described phosphorylation buffer (140 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 10 mM Tris-HCl, 20 μM MgATP), in the presence of 0.5 mM CaCl₂ or 1 mM EDTA [30], at room temperature. When purified CFTR was used, it was diluted in phosphorylation buffer in order to remove detergent as previously described [30]. Results obtained with CFTR and CFTR combined with annexin V (5 μg of each protein per well), in the presence of CaCl₂ or EDTA were compared with those using buffer alone (negative control). Experiments were also performed using the immunoprecipitated CFTR, in the presence of 0.5 mM CaCl₂ or 1 mM EDTA and the results were compared with that of buffer alone. The linearity of the luminescent response was tested during each experiment using 5 and 10 μg Kemptide according to the manufacturer's instructions (Promega, France). Kemptide, which is a specific peptide substrate for cAMP-dependent PKA, was used as a positive control. Experiments were performed three times and the results indicate that annexin V is not involved in the phosphorylation (not shown).

Immunofluorescence and confocal microscopy

For immunofluorescence studies, stably transfected A549 cells expressing human CFTR were grown on coverslips. Cells were fixed (4% paraformaldehyde in phosphate buffer saline, overnight at 4°C), permeabilized in PBS containing 0.25% saponin solution (30 minutes at room temperature (RT)) and blocked in the presence of 1% BSA. Cells were further incubated with

monoclonal anti-CFTR antibodies (monoclonal, 1/100, MM13-4, RD) for three hours at room temperature. After successive washes in PBS, the cells were incubated with a secondary FITC-labelled anti-mouse antibody (1/100, Sigma). Controls in which first or secondary antibodies were omitted were performed. For siRNA experiments, immunofluorescence was performed 40 hours after transfection. Untransfected cells were used as a negative control. To label the actin cytoskeleton, the cells were incubated with phalloidin-FluoProbes®547 (1/40, Molecular Probes) according to the manufacturer's instructions.

Confocal microscopy was performed on 7 µm thick cryosections of normal human bronchi obtained from the Hôpital de la Cavale Blanche (Brest, France) following approval by the local ethics committee. Sections were incubated with monoclonal anti-CFTR (1/10; anti-C terminus MAB 25031, RD Systems) and polyclonal anti-annexin V antibodies (1/10; sc-1929, Santa Cruz Biotechnology) for three hours at room temperature. Sections were further incubated with FITC-labelled anti-mouse or TRITC-labelled anti-goat secondary antibodies (Interchim) and were observed using a confocal microscope (Zeiss). Negative controls, where primary, secondary or both antibodies were omitted, were performed.

Cell surface expression of CFTR by biotinylation

To estimate the presence of the CFTR protein in the plasma membranes of the cells after the altered or increased annexin V expression, the proteins of the cell surface were biotinylated. Four T75 cm² flasks of 90-95% confluent cells were washed three times with ice-cold PBS (pH 8.0) to remove any contaminating proteins. The cells were incubated in Sulfo-NHS-SS-Biotin solution (Pinpoint™ Cell Surface Protein Isolation Kit, Pierce, USA) for 30 min at 4°C, scraped and centrifuged 3 min at 500 x g. The pellet was suspended in lysis buffer (Tris-HCl pH 6.8 50 mM; NaCl 100 mM; Triton X-100 2% and antiprotease cocktail) and incubated for 30 min on ice. The cell lysates were clarified by centrifugation (10,000 x g for 2 min at 4°C) and biotinylated

proteins were isolated on Immobilized NeutrAvidin™ Gel (Pierce, USA). In control experiments we used A549 cells, which did not express the CFTR protein, and cells transfected with both the cDNA encoding CFTR and the siRNA or with both the cDNA encoding CFTR and annexin V. The CFTR protein was further detected by Western blotting as described above.

Relative quantification of annexin V transcripts

Total RNA was extracted using the RNeasy® Mini Kit (Qiagen). Its concentration and quality were assessed using an Agilent 2100 Bioanalyzer (Agilent) according to the manufacturer's instructions. Relative quantifications of annexin V transcripts were assessed in a one-step format (100 ng total RNA) using the QuantiTect® SYBR® Green RT-PCR Kit (Qiagen) with the ABI PRISM® 7700 sequence detection system. Annexin V-specific primers and amplification of annexin V were previously described [31]. The relative amounts of the transcripts were estimated by comparing amplification profiles of cells transfected with annexin V-silencing siRNAs and cells transfected with non-silencing siRNAs (negative control siRNA).

Each experiment was performed three times (not shown).

RESULTS

CFTR – annexin V interaction is Ca²⁺ - and ATP-dependent

To investigate the presence of both annexin V and CFTR in the same protein complex, we performed co-immunoprecipitation experiments using normal human tissues from the upper airways. In Fig. 1A, we show that CFTR was present in the immunoprecipitated complex, which was further used to investigate the presence of annexin V. A control experiment performed with proteins from cells which do not express the CFTR protein was negative (Fig. 1A), showing the specificity of this interaction. Another control experiment in which an irrelevant antibody directed against mouse Ig was linked to the beads was negative (not shown). Since calcium is involved in the properties of annexin V [32, 33, 22, 26], co-immunoprecipitation was performed

in the presence of Ca^{2+} ions, EGTA or EDTA, using previously described concentrations [27]. We found that more annexin V bound to CFTR in the presence of Ca^{2+} than in the presence of EDTA or EGTA, when equal amounts of co-immunoprecipitated complexes were loaded on a gel (Fig. 1B). No signal was observed in the negative control performed with cells which did not express the CFTR protein.

For overlay assays, the purity of the purified CFTR, NBD1 and of the commercially available annexin V protein was assessed by gel electrophoresis followed by NiAg staining, which is more sensitive than Coomassie blue staining and permitted purity assessment because a single band was seen (Fig. 2A, left panels). Their molecular weights were as expected. Protein purity was further assessed by Western blottings in which a single band was seen for each protein (Fig. 2A, right panels). The sharp band observed for the CFTR protein was likely due to the fact that it was purified and because the amount of loaded protein was lower than in usual Western blots.

In overlay assays, pure annexin V was subjected to SDS/PAGE and transferred to a PVDF membrane, which was incubated with either purified CFTR or NBD1. As shown in Fig. 2B, there was a direct interaction between annexin V and both CFTR and NBD1. An irrelevant protein (BSA) was used and ruled out non-specific interactions (Fig. 2B). As a conclusion, these results demonstrated a direct interaction between CFTR and annexin V, and between annexin V and the NBD1 domain of CFTR.

In SPR experiments, one protein is linked to a sensor chip while the other protein is injected over the linked protein. The interaction is observed using computerized analysis, as a curve (sensorgram) showing association, equilibrium and dissociation phases given as resonance units (RU) as a function of time (seconds). We first immobilized CFTR-His proteins on the NTA sensor chip and injected annexin V. We found that the RU values increased with the amount of

injected annexin V (Fig. 3A). The specificity of the binding was checked by injection of an irrelevant protein (BSA), which did not induce a signal (not shown). In further experiments, annexin V was injected on immobilized NBD1-His proteins. Ten seconds into the dissociation phase, the RU values obtained with NBD1 were 7.50, 9.46, 21.28 and 35.54 for 0.25, 0.50, 0.75 and 1 μM of annexin V, respectively (Fig. 3B). These data confirm that annexin V binds the NBD1 domain of the CFTR protein and are in accordance with the results obtained in the overlay assays.

We also studied the influence of Ca^{2+} on the NBD1-annexin V interaction. Considering that intracellular Ca^{2+} concentrations are 100 nM, but may peak at 1 μM , three different concentrations (100, 500 and 1000 nM) were used to study the interaction between NBD1 and annexin V. A calcium dependence was observed (Fig. 3C). As intracellular calcium is taken to be 500 nM, we used this single Ca^{2+} concentration to study the interaction in the presence of both Ca^{2+} and MgATP. As NBD1 binds ATP [30], the interaction between annexin V and NBD1 was studied in the presence of increasing amounts of MgATP. With 1 μM annexin V, the response reached a plateau above 1 mM MgATP (data not shown). Therefore this concentration was used subsequently. As shown in Fig. 3D, the presence of both Ca^{2+} (500 nM) and MgATP (1 mM) increased the apparent affinity of 1 μM annexin V for NBD1. The plotted curves (Fig. 3D) indicated that the bound annexin V peaked with both MgATP and Ca^{2+} (418.7 RU after 10 seconds), whereas this level was lower in the presence of Ca^{2+} alone (245.1 RU). At the same time-point, the levels obtained with annexin V alone or in the presence of MgATP were not significantly different (21.7 and 18.7 RU, respectively). A negative control was performed by injecting BSA over NBD1. EDTA and EGTA could not be used in the running buffer because they are known to inhibit nickel chelation which was used to link NBD1 to the sensor chip.

SPR analysis was performed as described in the Methods to determine the equilibrium dissociation constant (K_D) of NBD1 and annexin V in the absence and presence of both calcium and MgATP. Increasing concentrations of annexin V were injected over NBD1 and the resulting RU values were plotted against annexin V concentration (Fig. 3E). As shown in the insets in Fig. 3E, Scatchard analysis linearized the data from the saturation binding experiments. The K_D values were 4.3 nM and 1.6 nM in the absence and presence of both calcium and ATP, respectively, indicating that the dissociation was reduced in the presence of both calcium and ATP.

The interaction between annexin V and CFTR is at NBD1

A competition experiment showing that blocking the NBD1 binding site of annexin V with the competitive CPX was performed using SPR. CPX was injected on the immobilized CFTR protein to block all the NBD1 sites and annexin V was injected. As shown in Fig. 4A, CPX inhibited the annexin V – CFTR interaction. We concluded that NBD1 was the single annexin V binding site in CFTR. The interaction between annexin V and the delF508-CFTR protein was also studied. As shown in Fig. 4B, the mutation did not alter the annexin V interaction. Using the delF508-CFTR protein, the SPR experiment with CPX was performed and we showed that CPX inhibited the annexin V – delF508 CFTR protein interaction (Fig. 4C). From these experiments we concluded that NBD1 is the single annexin V binding site in the wild-type CFTR and in the delF508-CFTR protein. Furthermore, the delF508 mutation did not alter the annexin V – CFTR interaction.

Annexin V–CFTR interaction is necessary for normal CFTR channel function

The physiological relevance of the interaction was studied. Since annexin V and CFTR interact, we hypothesized that this interaction has functional implications and that a reduced amount of annexin V in cells could modify CFTR function. Using macroscopic Cl^- flux through the CFTR channel, and SPQ fluorescent indicator, we first noticed that the expressed CFTR was

functional in our cells, whereas no Cl^- flux was observed in untransfected cells. This was in accordance with the immunofluorescence and Western blottings performed to validate the cell model. We compared the results obtained with A549 cells, A549 cells stably expressing CFTR, and A549 cells stably expressing CFTR in which annexin V expression was inhibited by siRNA transfection. Western blotting 0, 24, 40 and 72 hours after transfection with siRNA directed against annexin V indicated that its protein level was minimal at 40 hours (Fig. 5A). At this time-point, relative quantification of the annexin V mRNA showed it was reduced by 69% (+/-10%, data not shown). As annexin V is an actin-binding protein, the use of siRNA directed against annexin V could alter actin accumulation which could lead to modified chloride channel function of CFTR protein. Therefore, the level of actin protein was checked by Western blot. As shown in Fig. 5A, actin expression was not modified during the same time-course, indicating the specificity of the siRNA. The use of an irrelevant scrambled siRNA did not modify the amounts of either annexin V or actin, further indicating a specific effect of the siRNA. As shown in Fig. 5B, SPQ experiments indicated that inhibition of annexin V reduced Cl^- efflux via CFTR. The correlation between annexin V down-expression and CFTR activity was studied 24, 40 and 72 hours after siRNA transfection. The drop (between 0 and 40 hours after siRNA transfection) and then the increase (at 72 hours after transfection) in annexin V expression (Fig. 5A) was associated with a drop and then an increase in CFTR chloride channel function (Fig. 5C). Nevertheless, the chloride flux did not reach its initial values at 72 hours after transfection, perhaps because the molecular machinery needed to be active for longer. Controls with the siRNA buffer alone or with the scrambled siRNA did not impair the CFTR chloride channel function (Fig. 5C). We concluded that annexin V is necessary for normal CFTR channel function and that the effect of reducing the annexin V level was specific. During the SPQ experiments, the cell number in each microscopic field and the cell morphology were assessed. Because they were

identical to those of cells not transfected with siRNA, we also concluded that cell viability was not modified in the presence of siRNA. Cell viability was further assessed using trypan blue (not shown).

The effect of annexin V overexpression on the chloride channel function of CFTR was also studied. Annexin V expression was assessed at increasing time-points after the transfection (not shown). As shown in Fig 6A, the expression was increased 24 hours after the transfection ($69.8 \pm 0.8\%$), compared with controls. Actin expression was not modified at this time-point. The involvement of annexin V overexpression on the chloride channel function of CFTR was assessed by SPQ and was compared with that of cells which transfected with the empty vector. As shown in Fig 6B, CFTR function was increased when annexin V expression was increased.

Therefore, the down-regulation of annexin V reduced CFTR function, which increased when annexin V was overexpressed.

Cell morphology, actin and CFTR localization when annexin V expression is modulated

As annexin V is an actin-binding protein, and because actin is necessary for normal CFTR function, cell morphology as well as actin and CFTR distribution were studied by co-immunofluorescence in A549 cells 40 hours after transfection with siRNA directed against annexin V (Fig. 7). No difference was observed in cellular morphology, actin distribution or actin content by comparison with a control without siRNA (Fig. 7). CFTR distribution was modified by the presence of siRNA directed against annexin V. As shown in Fig. 7, CFTR protein was mainly detected in membranes in the absence of the siRNA directed against annexin V. In cells transfected with siRNA, CFTR labelling was diffuse and the membranes were not labelled anymore. We concluded that decreased annexin V expression led to reduced membrane localization of CFTR protein.

CFTR localization was further studied in biochemical assays. The membrane proteins

were labelled and purified in order to assess the presence of CFTR protein. As shown in Fig. 8A, CFTR was not present in the cellular membranes in cells that were not transfected with the cDNA encoding CFTR. In CFTR-expressing cells, decreased annexin V expression induced a loss of membrane localization of CFTR, whereas it was observed when a scrambled siRNA was used. The same experiment was performed when annexin V was overexpressed, and an increased localization of the CFTR protein within the membranes was observed (Fig. 8B). The decreased annexin V expression induced a total loss of CFTR in the membranes, suggesting the importance of annexin V in integration of CFTR within membranes. This result was in accordance with the immunofluorescent study (Fig. 7).

CFTR and annexin V distribution in human bronchial tissues

We found that annexin V could bind the CFTR protein. Therefore, we investigated the co-distribution of both proteins within cells in normal human tissues. Double staining of CFTR and annexin V was performed in normal human bronchi (Fig. 9A). In confocal experiments, CFTR was mainly observed in the membranes of epithelial cells from normal bronchi, with a stronger signal in apical membranes (Fig. 9A, left panel). In a study using CFTR antibodies, we previously observed this apical labelling (34). Some annexin V labelling was seen in the cytosol close to the apical membrane (Fig. 9A, middle panel). To our knowledge, this is the first time that annexin V localization has been described in human bronchial cells. In Fig. 9A (right panel), the merged images indicate that both proteins are partially distributed close to the apical region of normal human bronchial cells with interacting regions. Because CFTR is integrated inside the apical membranes and because annexin V was observed under the membranes since it is not an integrated protein, the co-distribution was partial. This was further assessed in an experiment performed with another tissue when a region where annexin V and CFTR were co-distributed was observed at high magnification (Fig. 9B).

DISCUSSION

Based on the properties of both annexin V and CFTR, and on the fact that annexin V is overexpressed in CF epithelial cells, we investigated the role of annexin V in CFTR-expressing cells. We hypothesized that annexin V may be involved in CFTR function. This hypothesis was reinforced by the previously described involvement of annexin I in CF [35], by the interaction between annexin V with membrane protein and with some exchanger [21] and by the modulated expression of annexin V in CF [16].

Co-immunoprecipitation using normal human tissues showed that annexin V and CFTR are present in the same protein complex. This led us to investigate whether the interaction is direct and to determine which CFTR domain is involved. Because NBD1 and annexin V may interact with PS [17, 19, 20], we hypothesized that they could be co-distributed and interact. Using purified NBD1, we showed a direct interaction between annexin V and NBD1 which is involved in CFTR chloride channel function and in most of the CFTR mutations. Further studies of the annexin V-NBD1 interaction were performed using the previously described SPR technology [36]. Because ATP binding is a feature of NBD1, and as annexins are known to be involved in PLA2 inhibition, membrane trafficking and exocytosis regulation, cytoskeletal protein binding, transmembrane channel regulation and intracellular signalling in a Ca^{2+} -dependent manner [32, 33, 37], we studied the involvement of MgATP and Ca^{2+} in the formation of the annexin V-CFTR complex. Our results indicate that the association between annexin V and CFTR is Ca^{2+} - and ATP- dependent, and the calculated dissociation constants were in accordance with our biochemical results. In the presence of Ca^{2+} and ATP, the interaction between NBD1 and annexin V was characterized by a K_D value of 1.6 nM. The interaction between annexin V and glycosaminoglycan was previously studied using SPR [38] and the Ca^{+2} dependence of this binding afforded a lower K_D value than the one we observed. Therefore, we confirm the

importance of the Ca^{+2} dependence in annexin V function and show that it may function mainly inside the cells. This point is further reinforced by the previously described interaction of annexin V with the sodium-calcium exchanger and caveolin 3 [21], which indicate that annexin V may modulate ionic homeostasis in cells.

At this point our work was descriptive, and we then studied the physiological implications of the interaction and showed that annexin V is involved in normal CFTR Cl^- channel function. Reduced annexin V expression lowered chloride channel activity of the CFTR protein, whereas annexin V overexpression led to an increased chloride flux. This indicated that annexin V is necessary for normal CFTR-mediated chloride channel function. This result, together with the described annexin V overexpression in CF patients, confirms the previous hypothesis assessing that annexin V may compensate the CFTR defect in CF [16].

Annexin V binds to actin [15, 33], which is involved in cAMP-dependent CFTR activation [39, 40]. Since the cellular actin organization and level are not altered in our cells, we conclude that the decreased Cl^- channel function is due to the decreased annexin V expression through a direct interaction with CFTR.

The mechanism by which annexin V expression could modify CFTR function was studied and two hypotheses were tested. First, we hypothesized that annexin V could be involved in CFTR membrane localization and in phosphorylation of CFTR. Therefore, we investigated the involvement of annexin V in the targeting of CFTR to the cell membrane and showed that the localization of CFTR was correlated with annexin V expression. Immunofluorescence and biochemical studies indicate that the presence of the CFTR protein in the membranes is dependent on the protein expression of annexin V. Our second hypothesis was an involvement of annexin V in CFTR phosphorylation. However, siRNA directed against annexin V did not alter CFTR phosphorylation (not shown). Furthermore, this second hypothesis was ruled out because it

was recently shown that the depletion of endogenous annexin V with siRNA inhibits delta-PKC translocation [41]. Also, annexin V inhibition of PKC through sequestering of its substrate is largely documented and the decreased annexin V expression in our experiments could in turn lead to increased PKC activation, which would not explain the decreased function of CFTR.

Our experiments do not rule out the involvement of intracellular calcium, which may be modulated by annexin V. Annexin V is a calcium-binding protein which exhibits calcium channel activity [33, 42, 43] and modulation of its expression could modify intracellular calcium, which is known to modulate CFTR chloride channel activity [44, 45].

The relevance of our findings was investigated *in vivo*, using co-immunoprecipitations in human tissues. Our confocal micrographs showing partial co-distribution of annexin V and CFTR in human normal bronchi are similar to those reported for CFTR-associated protein 70 [46]. This partial co-distribution can be explained because annexin V is mainly found under the apical membranes and is not a membrane protein, and because CFTR is a membrane-spanning protein.

The present results are of importance because the quaternary structure of proteins is of crucial importance for both their maturation and normal function [47]. It is therefore essential to identify new CFTR partners that may be involved in the genotype-phenotype relationship in CF. We focused on annexin V because it has recently been shown to be one of the most abundant proteins in CF cells [48], and have shown for the first time its functional interaction with CFTR. Because the most frequent CF-causing mutation is a deletion in NBD1 (delF508), it can be hypothesized that the interaction between CFTR and annexin V might be impeded, increasing the lethal effect of this mutation. Nevertheless, our experiments indicate that the interaction is not impeded by this mutation. According to the present results, the previously described overexpression of annexin V in CF can be seen as a cellular response, which tends to compensate for the CFTR defect, as proposed by Della Gaspera and co-workers [16].

Besides CF, we report new information regarding the function of annexin V in cells. Our results showing a direct interaction between annexin V and CFTR, taken together with recent data showing an association between annexin A5 and cardiac Na⁽⁺⁾/Ca⁽²⁺⁾ exchanger and between annexin V and caveolin [21], indicate that annexin V binds a membrane protein. Because annexin V is likely to be secreted together with lung lamellar body, which is thought to be an intracellular store of pulmonary surfactant secreted by alveolar type II cells [49], a complex involvement in CF may be ascribed to annexin V.

Showing that annexin V is involved in the presence of the CFTR protein in the cell membrane and that it is therefore involved in the chloride flux via CFTR, the present work provides evidence for a new CFTR partner with functional implications and gives new information on the molecular pathophysiology of CF. Furthermore, we show that annexin V overexpression leads to increased chloride channel function through CFTR. Therefore, annexin V may be seen as a new potential therapeutic target in CF.

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LEGENDS TO FIGURES

Figure 1

Detection of annexin V in CFTR co-immunoprecipitated complexes from normal human tissues.

(A) Magnetic beads were coated with the anti-CFTR antibodies and incubated with protein extracts from normal human tissues (upper airways). Complexes were subjected to SDS/PAGE (7.5%) and transferred onto a membrane which was incubated with anti-CFTR antibodies. CFTR

is detected (170 kDa) whereas it is not present when the beads were incubated with cells not expressing CFTR (A549). **(B)** Example of co-immunoprecipitations performed in the presence of calcium (CaCl_2 , 10^{-5} M), in the presence of EGTA (10^{-3} M) and in the presence of EDTA (10^{-3} M). Samples were resolved by a 12% gel electrophoresis and annexin V (35 kDa) was detected by Western blotting. The observed signal representing the amount of annexin V in the immunoprecipitated complexes is more intense in the presence of calcium than in the presence of EDTA or EGTA. Therefore, annexin V and CFTR are present in the same complex and the interaction is calcium-dependent. No signal is observed in the negative control performed with proteins extracted from A549 cells not transfected with the cDNA encoding CFTR.

Figure 2

Direct interaction between annexin V and the NBD1 of CFTR. **(A, left panels)** Purity of CFTR (170 kD), NBD1 (25 kD) and annexin V (35 kD) was assessed by gel electrophoresis (7%, 12% and 12%, respectively) of 2 μg of proteins and NiAg staining. A single band was observed for each protein. Western blotting (right panels) showed that all the proteins were pure. **(B)** In overlay experiments, 3 μg of pure annexin V was subjected to SDS/PAGE (12%) and transferred onto a membrane which was incubated with either purified CFTR or purified NBD1. Using specific antibodies, the detection of CFTR and NBD1 at 35 kD, corresponding to the molecular weight of annexin V, showed a direct interaction between annexin V and both CFTR and NBD1. The specificity of this interaction was observed using an irrelevant protein (BSA). The corresponding Coomassie blue staining of the membrane shows the amounts of the loaded proteins.

Figure 3

Study of the direct interaction between annexin V and CFTR and study of the positive role of calcium and ATP by SPR. **(A)** Example of sensorgram obtained when different amounts of

annexin V were injected over the immobilized CFTR protein. **(B)** Example of sensorgram obtained when annexin V (0.25, 0.50, 0.75 and 1 μM) was injected over the immobilized NBD1. **(C)** Effect of Ca^{2+} on annexin V (1 μM)–NBD1 binding. The figure shows an example of a sensorgram obtained in the presence of 100, 500 and 1000 nM Ca^{2+} , showing the calcium dependence of the interaction. **(D)** Example of sensorgram obtained when 1 μM annexin V was injected on the immobilized NBD1, in the presence of 1 mM ATP or 500 nM Ca^{2+} or both ATP and Ca^{2+} . The highest level of bound annexin V was obtained in the presence of both ATP and Ca^{2+} . **(E)** Determination of the dissociation constant of NBD1–annexin V binding in the absence and presence of 500 nM Ca^{2+} and 1 mM MgATP. Plots of the equilibrium response (R_{eq} in RU) versus annexin V concentration were determined when the injection curve showed a plateau. Insets: Scatchard analysis linearizing the data from the saturation binding experiments, to determine K_{Ds} . The R^2 values for the linear fit are indicated. BSA was used as a negative control in each experiment.

Figure 4

Inhibition experiment using SPR. **(A)** Example of sensorgram obtained when CPX was injected on the immobilized CFTR to block NBD1 followed by the injection of 1 μM annexin V, in the presence of 1 mM ATP and 500 nM Ca^{2+} . The last injection of CPX shows that all the NBD1 sites were blocked because the response is not increased. The injection of annexin V gave no response indicating that CPX inhibited the interaction with CFTR. **(B)** Example of sensorgram obtained when annexin V (0.25, 0.50, 0.75 and 1 μM) was injected over the immobilized delF508-CFTR in the presence of 1 mM ATP and 500 nM Ca^{2+} . The response is comparable to that obtained with the wild type CFTR indicating a direct annexin V – delF508-CFTR interaction. **(C)** The same experiment as in (A) was performed using the delF508-CFTR protein.

The injection of annexin V gave no response indicating that CPX inhibited the interaction with delF508-CFTR.

Figure 5

Annexin V is necessary for CFTR chloride channel activity. **(A)** Western blot showing the expression of annexin V (upper panel) and actin (lower panel) in the presence of siRNA directed against GFP and 0, 24, 40 and 72 hours after transfection of the siRNA directed against annexin V. Twenty μg of total protein were loaded on the gel. Annexin V and actin were detected on the same membrane. Pure annexin V was used as a control (1 μg). The Coomassie blue staining of the membrane (right panel) indicates that the amount of loaded protein is the same in all lanes. Histograms represent the relative quantitation of annexin V and actin. The actin level was not modified, whereas the level of annexin V was minimal 40 hours after siRNA transfection. **(B)** Macroscopic Cl^- flux experiments. The curves from the SPQ experiments reflect the activity of CFTR as a chloride channel on forskolin activation. The upper curve (black circles) was obtained with stably transfected A549 cells expressing CFTR (n=7). The middle curve (white triangles) was obtained with untransfected cells (n=12). Finally, the lower curve (white circles) reflects the activity of the CFTR protein in the presence of siRNA against annexin V (n=6) which abolished Cl^- flux ($p < 0.001$) giving results similar to those of cells without CFTR. A cAMP-stimulating mixture of IBMX (100 μM) and forskolin (10 μM) was added at the indicated time. F/F_0 is the normalized fluorescence and bars represent SEM. **(C)** Time dependence between CFTR activity and siRNA post-transfection time. Chloride flux was measured without siRNA transfection (white circles) and 24 (white triangles), 40 (black lozenges) and 72 hours (white squares) after siRNA transfection. The chloride flux was minimal when the expression of annexin V was decreased. The transfection buffer alone or the scrambled siRNA had no effect upon chloride flux

(not shown). Bars represent standard deviations (SDs) from at least five experiments.

Figure 6

Effect of the overexpression of annexin V upon the chloride channel function of CFTR. **A.** Example of Western blot showing the expression of annexin V (upper panel) and actin (lower panel) when the cells are transfected with the empty pcDNA3.1 vector or with the vector in which the cDNA encoding annexin V is inserted (24 hours post-transfection, upper panel). In the presence of the cDNA, annexin V is overexpressed in the cells, whereas the actin level is not modified (lower panel). Ten μg of total protein were loaded on the gel. **B.** Macroscopic Cl^- flux experiments. The curves from the SPQ experiments were recorded as described above. The upper curves were obtained with stably transfected A549 cells expressing CFTR in the presence of the empty pcDNA3.1 vector (white squares) and in the presence of the vector in which the cDNA encoding annexin V was ligated (black squares). The lower curves were obtained with untransfected cells in the presence of the empty pcDNA3.1 vector (white circles) and in the presence of the vector in which the cDNA encoding annexin V was ligated (black circles). The chloride channel activity of CFTR was increased when annexin V was overexpressed. F/F_0 is the normalized fluorescence and bars represent SEM ($n = 4$).

Figure 7

Cell morphology and CFTR distribution following annexin V silencing. Immunostaining of CFTR and actin (phalloidin) was performed in the absence or presence of siRNA (40 hours). Two examples are shown. In the absence of the siRNA, the CFTR was seen in the membranes of the cells (white arrows, upper panel). In the presence of the siRNA, the CFTR labelling was diffuse and was not seen in the membrane (middle panel). In the right panel, the nuclei were stained with DAPI. Actin (red, lower panel) distributions did not differ between the two conditions, showing that the siRNA had no effect on cell morphology.

Figure 8

CFTR protein detection at the cell surface of A549 cells when annexin V expression is modulated. Membrane proteins were biotinylated and CFTR was detected by Western blotting (7.5% gel electrophoresis). **A.** Whereas the CFTR protein is observed in the cell membrane of CFTR-expressing cells in the presence of the scrambled siRNA, it is not seen when cells are transfected with the siRNA directed against annexin V. Untransfected cells, which did not express CFTR, were used as negative control. The reduced annexin V expression induced a loss of CFTR in the membranes. **B.** CFTR is detected in the membrane of the cells that were transfected with both the cDNA encoding CFTR and the empty pcDNA3.1 vector. In CFTR-expressing cells, the CFTR was increased in the presence of the pcDNA3.1 vector in which the annexin V cDNA was ligated. Untransfected cells, which did not express CFTR, were used as negative control. Therefore, annexin V overexpression induces increased CFTR localization in the membranes.

Figure 9

Example of the distribution of CFTR and annexin V in normal human bronchi observed by confocal microscopy. **A.** CFTR (FITC, green) is mainly observed in apical membranes of epithelial cells (left panel). Some annexin V (TRITC, red) is apically localized, close to the membrane (middle panel). Merged images of CFTR and annexin V show that both proteins are partially co-distributed in the apical membrane (yellow). First and second lines represent increasing magnifications. The third line represents an increased magnification of the inset of the second line. **B.** High magnification of a image of merged labelling of both CFTR and annexin V in another human bronchi. Whereas CFTR is in the apical membrane of the cells, annexin V is seen under the membrane because it is not an integrated protein. The co-distribution of both proteins (yellow) is indicated by arrows. L: Lumen of the bronchi.

Figure 1

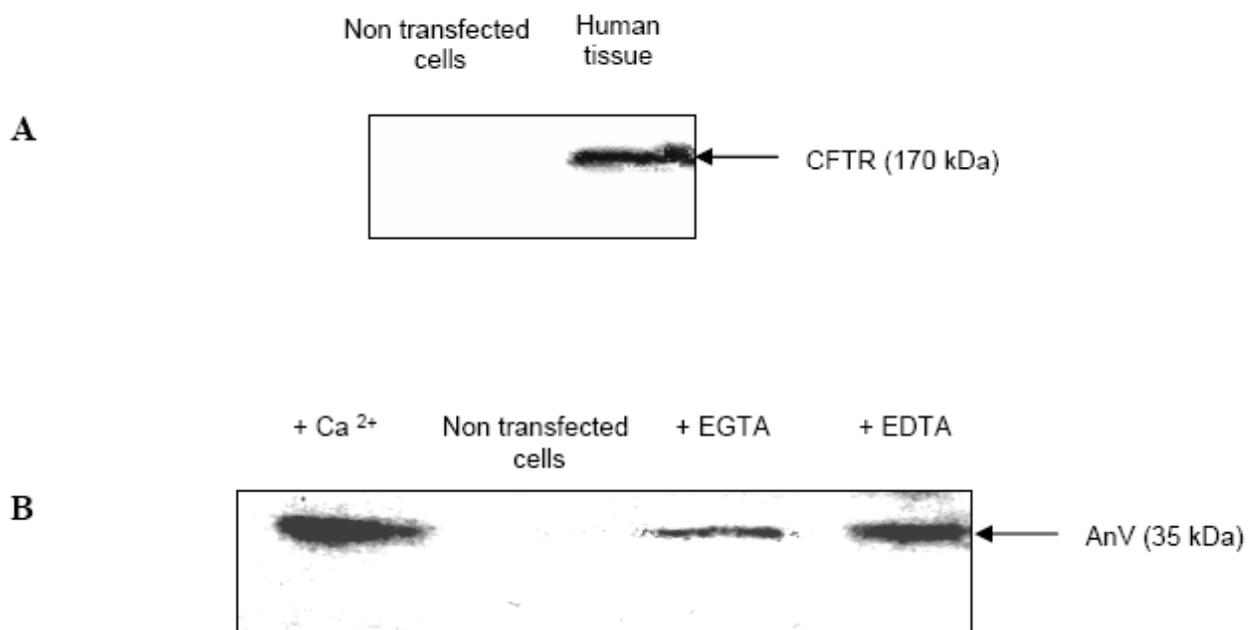


Figure 2

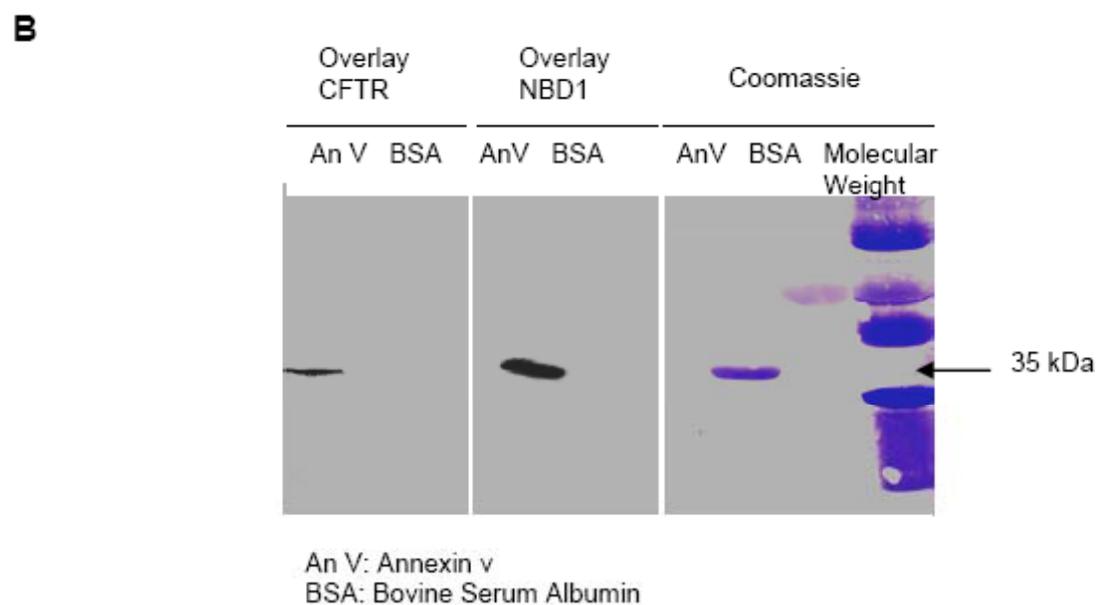
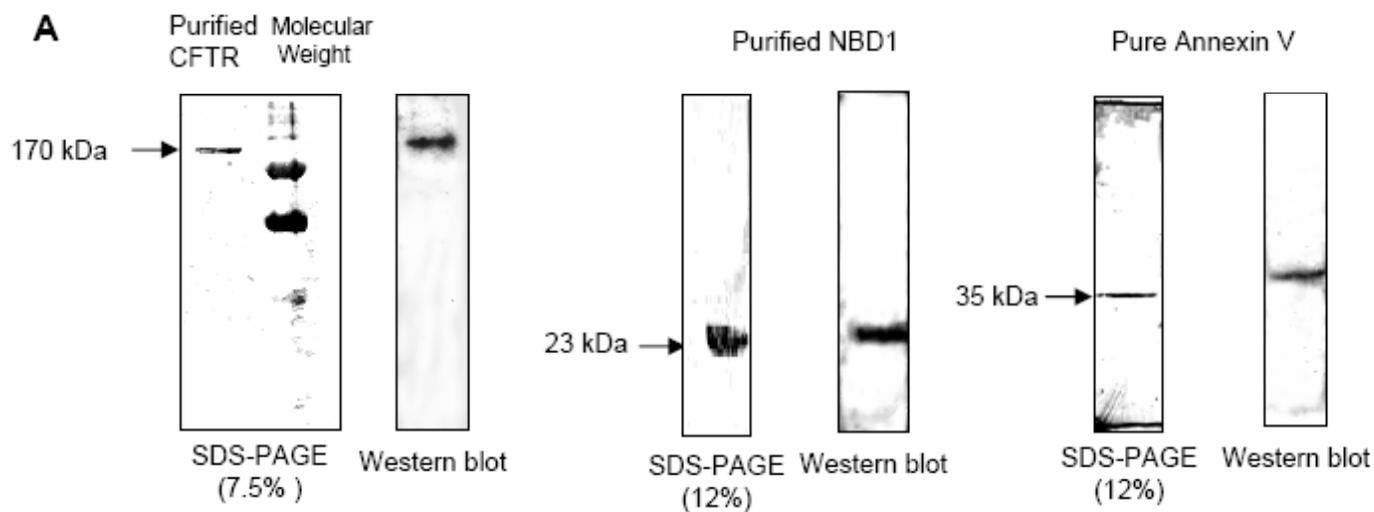
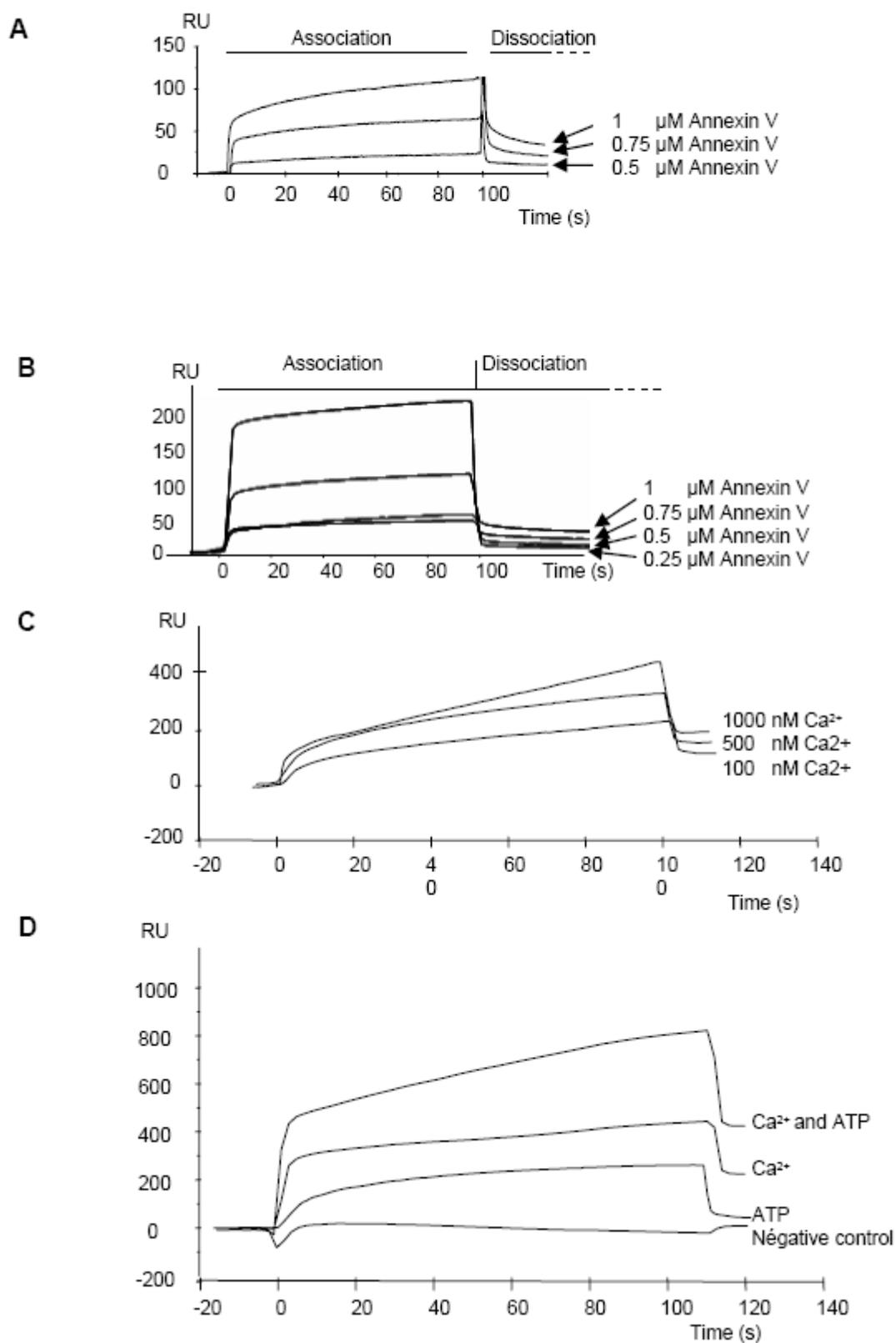
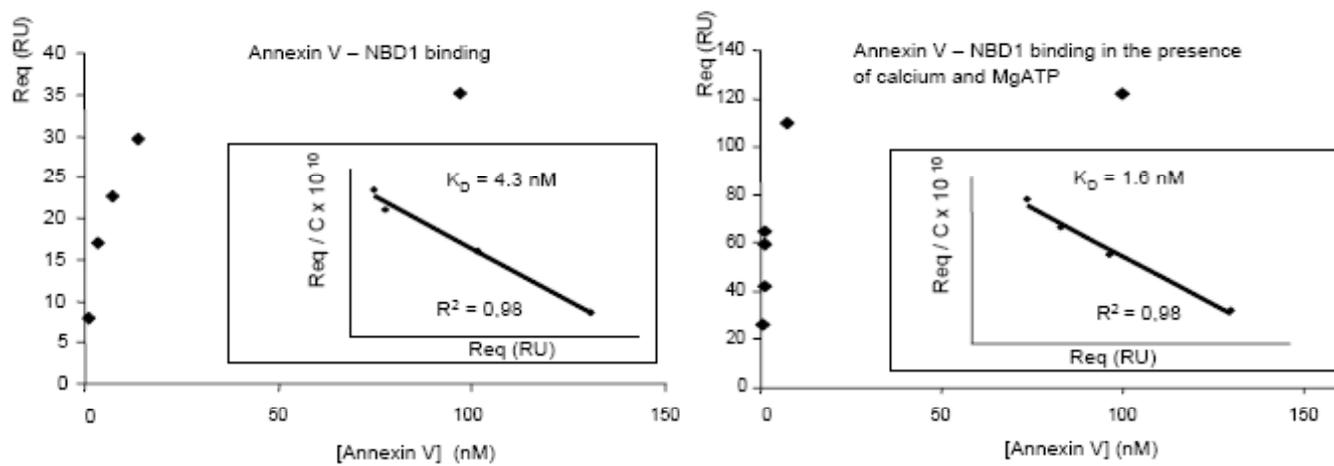


Figure 3



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Figure 4

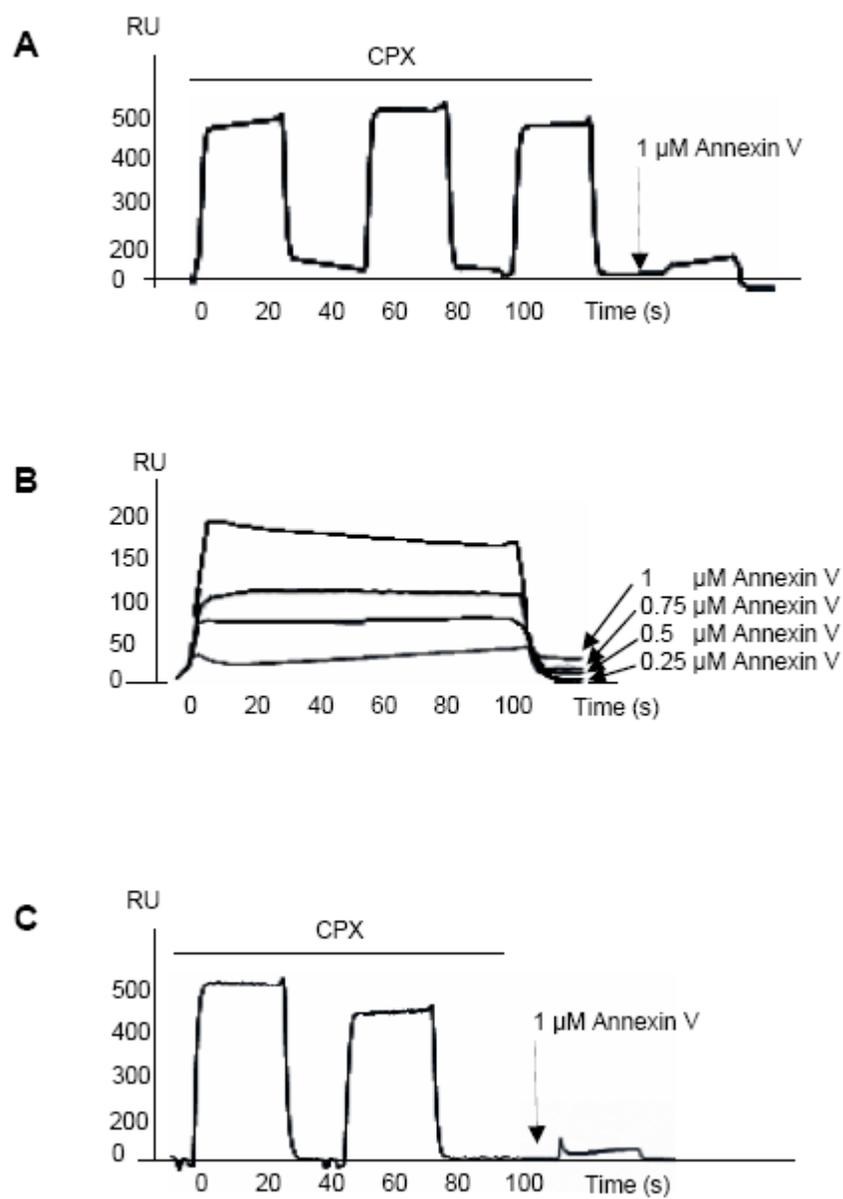


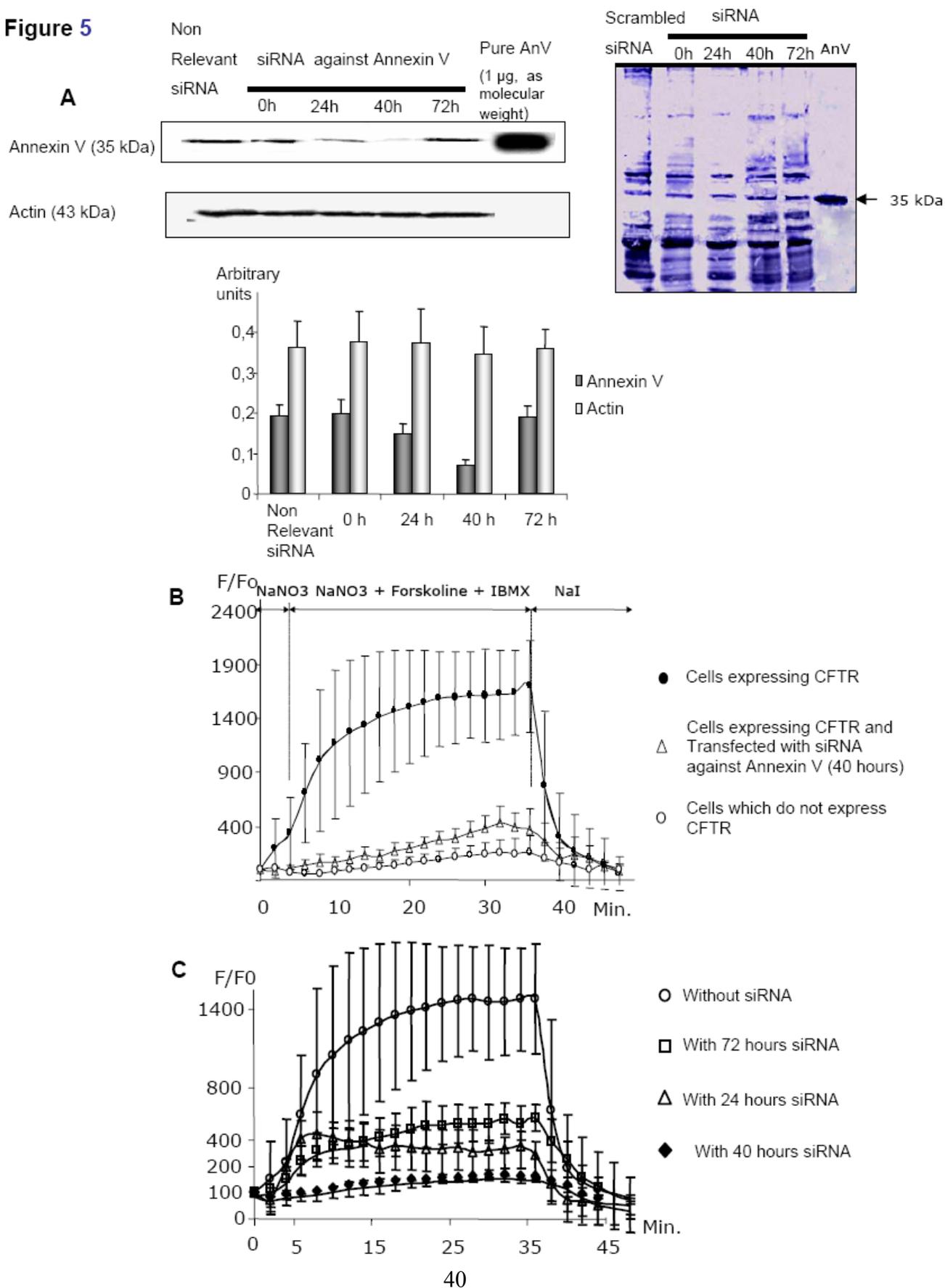
Figure 5

Figure 6

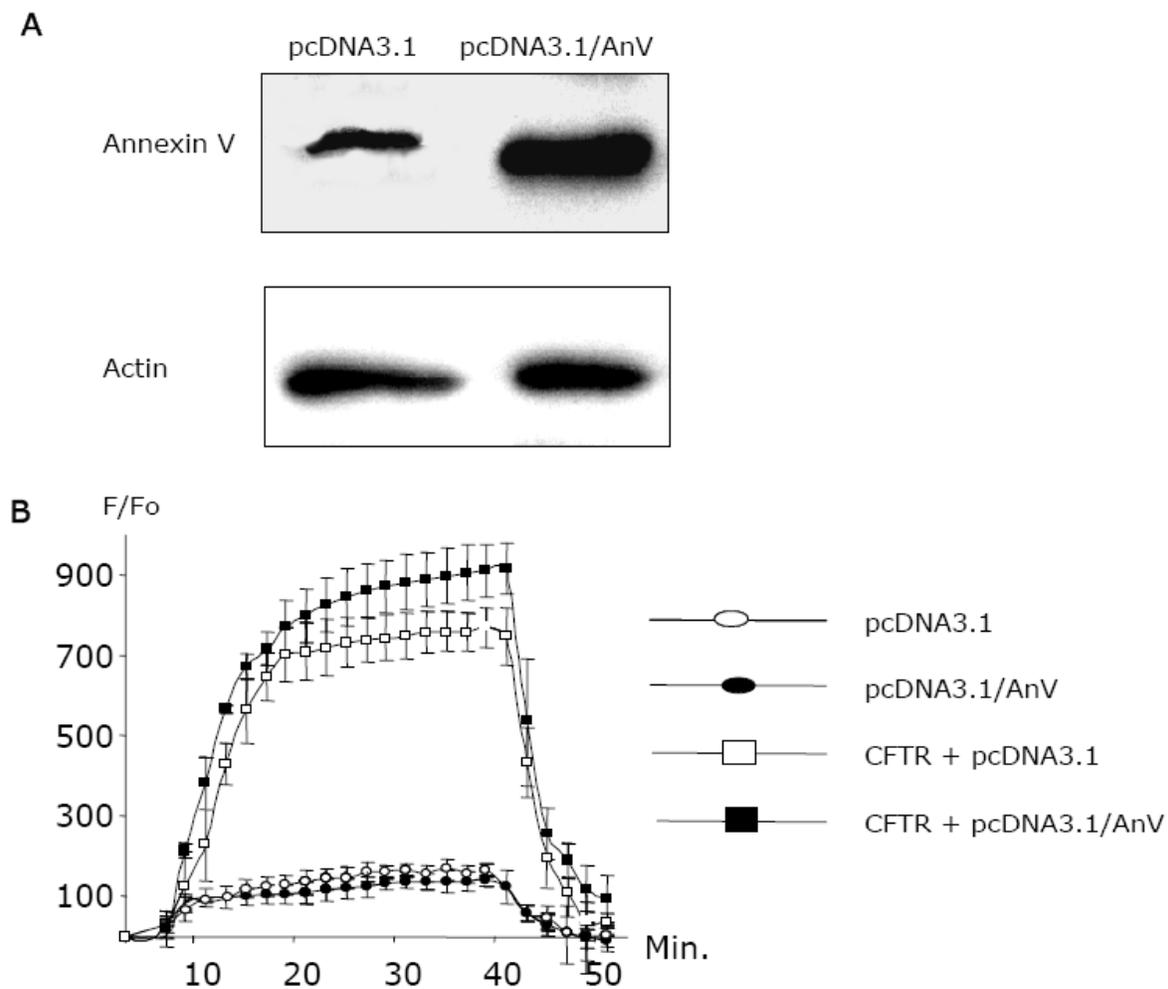


Figure 7

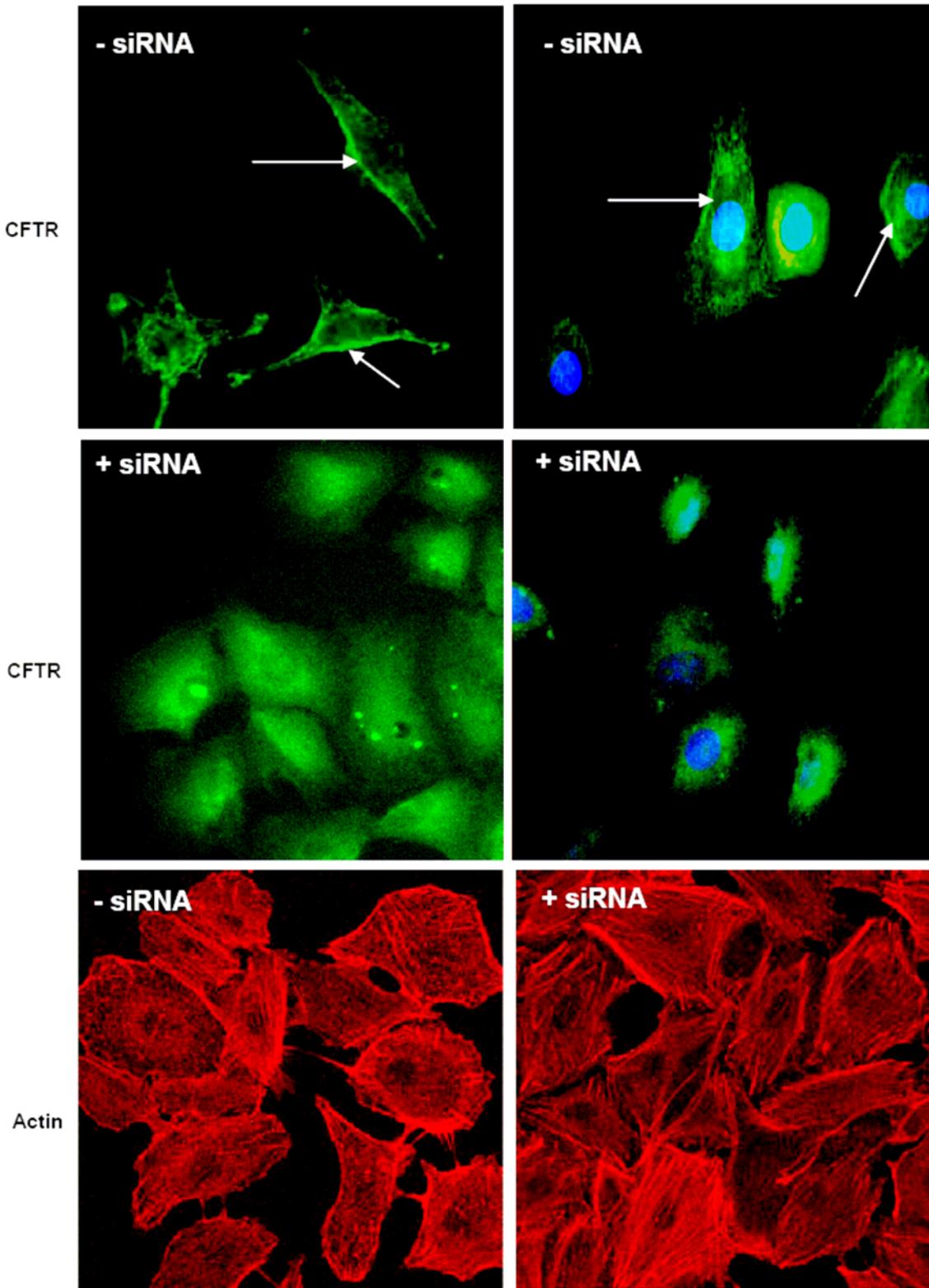


Figure 8

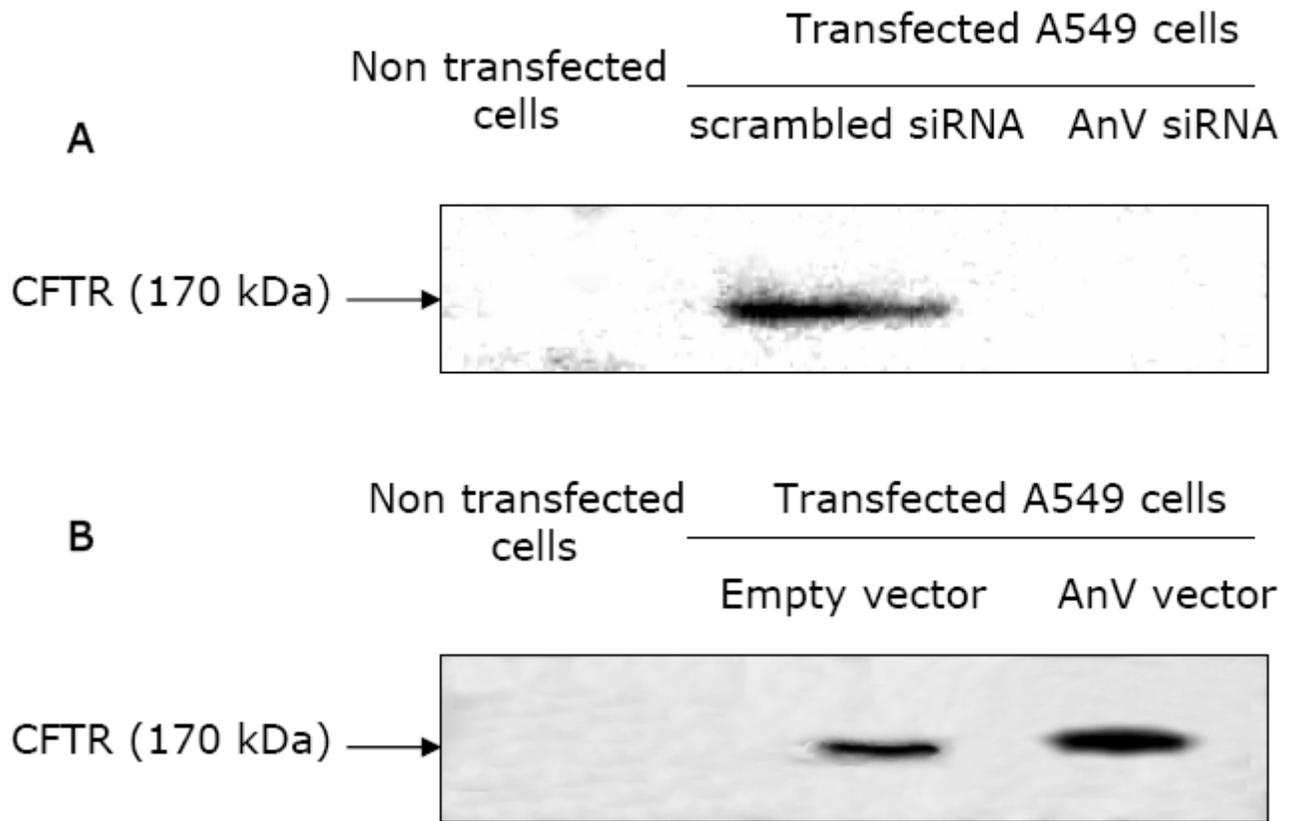
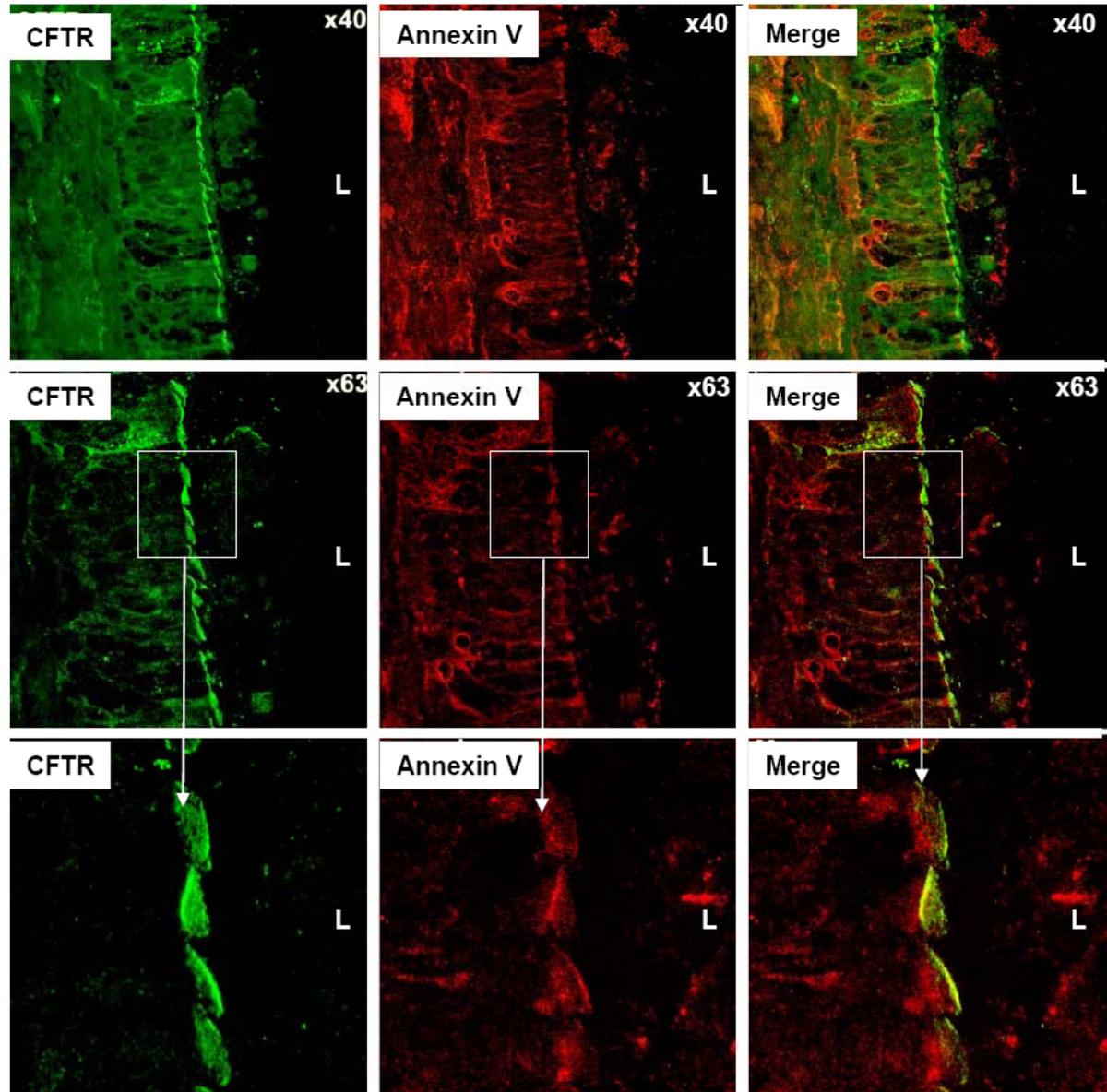


Figure 9

A



B

