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3 **Rapid detection of the mature form of Cystic Fibrosis Transmembrane Regulator by**
4 **Surface Plasmon Resonance**
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53 *Keywords:* Surface plasmon resonance, cystic fibrosis transmembrane conductance regulator.
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56 *Abbreviations:* Surface plasmon resonance, SPR; resonance units, RU; cystic fibrosis
57 transmembrane conductance regulator, CFTR.
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Abstract

Cystic fibrosis is the most common lethal autosomal recessive disease in the Caucasian population, and is due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The CFTR protein functions as a chloride channel. Electrophoretic analysis shows that normal CFTR exists as three different molecular weight forms of 127, 131 and 170 kDa (band A, B and C, respectively) representing different glycosylation forms. Band A is the non glycosylated form of CFTR, band B is the core glycosylated CFTR and band C is the mature form of CFTR with complex glycosylation. The glycosylation state of CFTR is representative of its maturation and is an important marker of the protein processing and function. The most common mutation in CF is a missing phenylalanine at position 508 (F508del-CFTR). The misfolded F508del-CFTR protein which exhibits an altered glycosylation, is observed as bands A and B only and does not traffic correctly to the plasma membrane. Laboratory experiments (SDS-PAGE, immunoblotting, glycosidase digestions, mobility shift of deglycosylated CFTR) aimed to assess the expression of CFTR and to depict which band is observed have been developed. Nevertheless, these experimental procedures are time consuming and poorly specific. The aim of the present study was to provide an easy, rapid and reproducible methodology to assess whether the CFTR protein within a protein extract is expressed and matured. We show here that surface plasmon resonance (SPR) permits a direct detection of the mature form of CFTR in crude cell lysates, providing a new tool to characterize CFTR in cells without any labelling or pre-treatment before cell lysis. The study of the effects of correctors for F508del-CFTR is the main task of many laboratories. Therefore, the proposed method is likely a useful tool for a rapid detection of mature CFTR in complex samples. We also show here that our method permits the characterization of CFTR in patients cell extracts in a minimum time.

1. Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population, and is due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1-3]. CFTR which is an ATP-binding cassette transporter, functions as a chloride (Cl⁻) channel [4-6] and comprises two hydrophobic core regions, two nucleotide-binding domains (NBDs) with ATP-binding activity [7] and a regulatory domain (R domain). Two N-linked glycosylation sites are present in the fourth extracellular loop of the normal CFTR protein (wt-CFTR, [2]). The newly synthesized wt-CFTR is rapidly degraded in the ER, as a core-glycosylated folding intermediate and depending on the expression system only 20 to 50 % of the protein attains maturation [8]. Electrophoretic analysis shows that wt-CFTR exists as three different molecular weight forms of 127, 131 and 170 kDa referred as bands A, B and C respectively [9]. These three bands represent different glycosylation forms of wt-CFTR. Band A is the non glycosylated form of CFTR, band B is the core glycosylated CFTR and band C is the mature form of CFTR with complex glycosylation [9]. The glycosylation state of CFTR is representative of its maturation and is an important marker of the protein processing and function [9].

The most common mutation in CF is a missing phenylalanine at position 508 (F508del-CFTR), which occurs in the first nucleotide-binding domain of the CFTR protein. The misfolded F508del-CFTR protein does not traffic correctly to the plasma membrane and is retained in the endoplasmic reticulum (ER) and degraded by proteasome [10-17]. This F508del-CFTR protein which is unable to traffic normally to the Golgi apparatus exhibit an altered glycosylation and is observed as bands A and B only [10, 18]. Beside F508del-CFTR, over 1940 mutations (www.genet.sickkids.on.ca/cftr/) have been identified in the CFTR gene and a classification of mutations by which different mechanisms induce CF has been proposed [20].

The appearance of the complex type N-linked oligosaccharides of the wt-CFTR and thus its glycosylation state is of main importance to characterize the maturation state of the protein. It is also important to assess whether a given mutation has an impact upon CFTR maturation and membrane localization. Therefore, laboratory experiments aimed to assess the expression of CFTR and to depict which band is observed have been developed, keeping in view that the C band correspond to a protein which is exported to membranes and is likely active as a Cl⁻ channel. Nevertheless, these experimental procedures are time consuming and poorly specific, needing SDS-PAGE and immunoblotting. To be fully sure of the

glycosylation state of CFTR, distinction between the core- and the complex-glycosylated CFTR need to be confirmed by glycosidase digestions by endoglycosidase H and peptide N-glycosidase F, the mobility shift of deglycosylated CFTR being observed by immunoblotting [12]. Because the glycosylation state of CFTR is correlated to its membrane localization and function, the aim of the present study was to provide an easy, rapid and reproducible methodology to assess whether the CFTR protein within a protein extract is matured or not. We show here that surface plasmon resonance (SPR, [19]) permits a direct detection of the mature form of CFTR in crude cell lysates, providing a new tool to characterize CFTR in cells without any labelling of pre-treatment before cell lysis.

2. Methods

2.1 Antibody and chemicals

CFTR proteins were probed using a monoclonal anti-CFTR antibody (24-1, R&D System). Wheat Germ Agglutinin (WGA, *Triticum vulgare*) was from Calbiochem (France). Sensor chip CM5, Amine coupling kit (*N*-hydroxysuccinimide, (NHS), *N*-ethyl-*N*-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC), ethanolamine (1 M, pH 8.5) and HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005 % surfactant P20 at pH 7.4) were obtained from GE Healthcare Bio-Sciences AB.

Purified wt-CFTR and F508del-CFTR proteins were a generous gift from Pr Robert Ford (University of Manchester, UK; [20]).

All experiments were performed at the *PurIProb* core facility (Inserm, UMR1078, Brest).

2.2. Ant-CFTR antibody and WGA immobilization

Real-time detection of CFTR in cell lysates was performed using the Biacore system (Biacore 3000; GE Healthcare) and its Control Software version 3.2. All injections were performed at 25 °C in HBS-P 1× running buffer (GE Healthcare). Biacore 3000 was set at 25 °C for all steps during the analytical process, and experimental data were collected at a medium rate. Biacore sensorgrams were analyzed using the BIAevaluation software. For each sample the indicated RU (Resonance Unit) value is the value on the active flow cell (FC, FC2 and FC4) minus the value of the reference FC (FC1 and FC3), 20 seconds after the beginning of the dissociation phase.

Binding of the CFTR antibody was performed according to Biacore recommendations on a CM5 sensor chips which is a carboxymethylated dextran matrix covalently attached to a gold surface. Antibody was immobilized on a CM5 biosensor chip using the Amine Coupling

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3 Kit (GE Healthcare). The active surface was activated with equal volumes of NHS and EDC
4 for 7 min with a 5 μ l/min flow. The binding events at a sensor surface induce changes in SPR
5 signal which are expressed in resonance units (RU). One RU is given to be equivalent to one
6 picogram of protein per square millimeter on the sensor surface. The surface was then blocked
7 with 1 M ethanolamine hydrochloride (pH 8.5) injection for 7 min with a 5 μ l/min flow. The
8 reference channel was activated with equal volumes of NHS and EDC and immediately
9 saturated with ethanolamine.
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12 WGA was immobilized on FC2 and the anti-CFTR antibody was immobilized on FC4.
13 FC1 and FC3 were their respective control FC. All proteins were diluted in HBS-EP buffer
14 and injected at a flow rate of 5 μ l/min. Injected volumes were 40 μ l and 10 μ l passed over the
15 FCs according to Biacore 3000 functioning. Absolute amounts of injected proteins are
16 indicated in figures, instead of concentrations, to give a more clear idea of the bound
17 quantities of proteins. All injections were performed at least in duplicate and BSA was used
18 as a negative control.
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20 **2.3 Cell cultures and protein extraction**

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22 Hela cells stably expressing either Wt-CFTR or F508del-CFTR were obtained and
23 characterized previously [21]. Wt-CFTR and F508del-CFTR expressing cells were collected
24 and homogenized in 2 mL of RIPA buffer. Lysates were ultra-centrifuged (32500 rpm, 30
25 min., 4°C) and pellets were resuspended in HBS-P 1 \times running buffer (GE Healthcare) and
26 injected over the sensorchip.
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29 In some experiments, proteins from nasal cells were used. Nasal cells were obtained
30 after brushing of healthy controls (n=3) and CF patients (n=3) which were informed and from
31 which a full consent was obtained. Cells were cultured as previously described [22]. In brief,
32 after nasal lavage with physiological saline buffer, a brushing was performed in both nostril,
33 along the tip of the inferior turbinate and the lateral nasal wall, by a gentle circular movement
34 for 1 minute, using a sterile brush (sterile endocervical brush, Diam 5.5, Laboratoire Gyneas,
35 Goussainville, France). Brushes were immersed in 1 ml of Ham's F12 containing Ultrosor G
36 2% (Lifescience) and an antibiotic/antimycotic solution and transported to the laboratory.
37 Epithelial cells were detached from the brushes by tweezing in a culture dish. Samples
38 obtained from the both nostril were pooled and centrifuged for 5 minutes (300 g) at room
39 temperature. The cell pellets were resuspended in 1 ml of SAGM (SAGM Bullekit, Lonza).
40 To improve cell adhesion, culture plates were coated with collagen from human placenta
41 (Type VI, Sigma Aldrich). When the cells were confluent, they were harvested and lysed in
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3 NP40 buffer (50 mM Tris-HCl at pH 7.4, 250 mM NaCl, 5mM EDTA, 50 mM NaF, 1mM
4 Na₃VO₄, 1% Nonidet P-40 and 0.02% NaN₃; NP40 Cell Lysis Buffer, Invitrogen).

6 **2.4 Statistical analysis**

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8 Student's t-test was used and differences were considered significant when $p < 0.05$ (*)
9 and $p < 0.0001$ (***)
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11 **3. Results**

12 Before final immobilization, the immobilization pH scouting test was performed in
13 order to determine the buffer to use for the antibody dilution. The suitable buffer for the
14 antibody was determined to be 10 mM acetate; pH 5.0 and the CFTR antibody was injected to
15 achieve 9509 RU. First, increasing amounts of purified non mutated CFTR and F508del-
16 CFTR proteins (0, 1, 2, 10, 20, 40, 50, 100, 500 and 1000ng) were injected on the anti-CFTR
17 antibody and sensorgrams were recorded. As shown in Fig. 1A, non mutated CFTR and
18 F508del CFTR proteins were recognized by the immobilized anti-CFTR. Nevertheless, a
19 difference was observed. When a same amount of the two proteins was injected, a higher RU
20 number was observed during the equilibrium and dissociation phases for the non mutated
21 CFTR. RU values were collected 20s after the beginning of the dissociation phase and were
22 plotted against the amount of proteins. 20s was chosen to represent the amount of bound
23 protein on the antibody, after washing, to remove any non-specifically bound material
24 contribution and to reach the equilibrium of the bound material. As shown in Fig. 1B, the
25 responses were linear for both proteins. Nevertheless, the response was significantly lower for
26 the F508del-CFTR protein, indicating that the affinity is likely lower for the mutated protein.
27 Indeed, the maximum response for 1000ng of proteins was 606 ± 12 RU and 375 ± 31 RU for
28 non mutated CFTR and F508del-CFTR, respectively. This difference is likely not due to the
29 mutation which corresponds to the deletion of a single amino acid.
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32 The pH scouting test indicated that WGA had to be diluted in 10 mM acetate buffer,
33 pH 4.5. Binding of WGA was performed as described above to achieve 5329 RU. The same
34 quantities of non mutated CFTR and F508del-CFTR proteins were injected on WGA. As
35 shown in Fig. 2A, they were recognized by the immobilized lectin but the responses were low
36 and the simple analysis of the sensorgrams was poorly informative despite a likely higher
37 response for the non mutated CFTR. Surprisingly, a positive response for the F508del-CFTR
38 protein was observed. RU values were then plotted against the amount of injected proteins. As
39 shown in Fig. 2B, two phases were observed, depending on the amount of injected proteins.
40 Above 50ng (Fig. 2B) the response for both proteins was linear and the level of the RU values
41 was significantly higher for the non mutated CFTR than for F508del-CFTR. Indeed, the
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3 maximal response for F508del-CFTR was only 15 ± 1.5 RU. It was also observed that the
4 slope of the curves were very different. The slope of the curve obtained by the injection of
5 F508del-CFTR protein was 0.0051, indicating a very low association of the protein on the
6 lectin, in function of the amount of injected protein. For low quantities of injected proteins
7 (ranging from 0 to 50ng), the curves were not linear. A specific fitting is therefore presented
8 in Fig. 2C. From the equations of the curves it was estimated that a positive response was
9 obtained above 3.27 and 0.3ng for non mutated CFTR and F508del-CFTR, respectively. This
10 calculation was in accordance with the experimental result. From the curves shown in Fig. 2B
11 and 2C it was concluded that for very low concentrations a response was observed for
12 F508del-CFTR but that the maximum was reach sooner than non mutated CFTR, with no
13 further increase of the response above 10ng. Apparent constants according to fitting model
14 1:1 ("Langmuir binding") were calculated and the K_D were of $1.3 \cdot 10^{-11}$ M and $< 2 \cdot 10^{-8}$ M for
15 Wt-CFTR and F508del-CFTR proteins, respectively. Whereas K_D values were different, the
16 k_D constants were close for both proteins ($1.7 \cdot 10^{-5} \cdot s^{-1}$ and $1 \cdot 10^{-5} \cdot s^{-1}$ for non mutated CFTR
17 and F508del-CFTR proteins, respectively). The final conclusion for this experiment was that
18 the binding of Wt-CFTR on WGA was higher and that the binding of F508del-CFTR protein
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31 Because the binding of the purified proteins on the anti-CFTR antibody does not
32 permit to discriminate the non mutated CFTR from the F508del-CFTR protein due to the
33 presence of its epitope on both proteins and because their binding on WGA did not follow a
34 binary mode, double injections were tested. First they were injected over the immobilized
35 antibody. Before the base line was reached, WGA was injected. According to the results
36 observed in Fig. 1, proteins associated with the antibody showed a higher response for the
37 normal protein (Fig. 3A). The injection of WGA showed a higher response on the non
38 mutated CFTR than on F508del-CFTR (Fig. 3A). Increasing amounts of proteins (0, 100, 500,
39 1000 and 1500ng) were injected and 2 μ g of WGA were injected on the proteins which were
40 immobilized on the antibody. Using the RU value before WGA injection as a base line, the
41 responses were collected 20s after the beginning of WGA dissociation. The RU values were
42 plotted against the amount of injected purified proteins. As shown in Fig. 3B (left panel), the
43 response due to WGA binding was increasing accordingly to the amount of bound non
44 mutated CFTR on the antibody. The response due to WGA binding on F508del-CFTR
45 remained low (< 2 RU). A response was only observed above 100ng of immobilized F508del-
46 CFTR and staid stable above 1000ng. The quantitation of the responses were further
47 performed when 2 μ g of purified proteins were injected on the immobilized antibody and
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3 when WGA was injected. As shown in Fig. 3B (right panel), the quantity of bound WGA was
4 significantly higher on the non mutated CFTR than on the F508del-CFTR. The opposite
5 experiment was performed. The purified proteins were injected over WGA and the anti-CFTR
6 antibody was injected. An example of the resulting sensorgram is shown in Fig. 4A. The
7 binding of the non mutated CFTR on WGA was higher than the one of F508del-CFTR.
8 Further injection of the antibody showed that indeed, WGA retains more non mutated CFTR
9 protein. This was confirmed by the injection of increasing quantities of protein over WGA
10 (Fig. 4B, left panel). The maximum binding of WGA on the F508del-CFTR was reached (<10
11 RU) with 500ng immobilized proteins onto the antibody whereas it was not for the non
12 mutated CFTR. The quantitation of the responses were further performed when 2 μ g of
13 purified proteins were injected on the immobilized WGA and when antibody was injected. As
14 shown in Fig. 4B (right panel), the quantity of bound antibody was significantly higher on the
15 non mutated CFTR than on F508del-CFTR. This indicated that more non mutated CFTR was
16 bound on WGA than F508del-CFTR.
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18 Further experiments were aimed to detect Wt-CFTR and F508del-CFTR and to detect
19 the mature form of the protein in crude protein extracts from cells.
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21 In a first set of experiments, stably transfected Hela cells expressing either Wt-CFTR
22 or F508del-CFTR were used. Total proteins from those cells were injected on FC4 on which
23 the anti-CFTR antibody was immobilized. As shown in Fig. 5A, both protein extract gave a
24 positive signal showing that the Wt-CFTR as well as the F508del-CFTR proteins were present
25 in the cell lysates. Nevertheless, as it was observed with purified proteins (Fig. 1), a higher
26 response was obtained with Wt-CFTR expressing cells. Various amount of protein extracts (0
27 to 2 μ g) were injected on the immobilized antibody and RU values were plotted against the
28 injected quantities (Fig. 5B). The upper panel of Fig. 5B shows the whole curves. Above
29 100ng of proteins a plateau phase was observed. In order to see the behaviour of the injections
30 below 100ng a second curve was plotted (Fig. 5B, lower panel). It was observed that for low
31 amount (<50ng) of injected proteins, there was no difference between the extracts of Wt-
32 CFTR and F508del-CFTR expressing cells were. From these injections it was concluded that
33 it is possible to detect the CFTR protein by the use of an immobilized antibody. Nevertheless,
34 because the antibody recognize Wt-CFTR and F508del-CFTR, the observed difference
35 between Wt-CFTR and F508del-CFTR expressing cells was not sufficient to discriminate the
36 the mature protein. Therefore, 0.5 μ g WGA was injected during the dissociation phase of the
37 whole cell extracts (Fig. 5C, left). A higher signal was observed for the Wt-CFTR expressing
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3 cells than for the F508del-CFTR expressing cells, corresponding to the glycosylated form of
4 the protein (mature form). The response due to the injection of WGA was further quantified
5 (Fig. 5C, right) to show that indeed, there was a difference between the proteins from the two
6 cell lines and to show that the observed difference was significant. To avoid the bias due to a
7 higher binding of proteins from Wt-CFTR expressing cells the base line was taken just before
8 the injection of WGA. Transfected Hela cells were then injected over the immobilized WGA.
9 As it is shown on the resulting sensorgrams (Fig. 6A), the difference between the proteins
10 extracted of Wt-CFTR expressing cells and F508del-CFTR expressing cells was important,
11 with RU values for the latest cells close to zero. These values were plotted against the amount
12 of injected proteins (Fig. 6B). The observation of the upper curve of Fig. 6B shows that below
13 100ng of injected proteins, the RU values were very low for the proteins from F508del-CFTR
14 expressing cells. Therefore, another curve was drawn for quantities ranging from 0 to 50ng
15 (Fig. 6B, lower panel). This curves show that the values of the protein extracts containing the
16 mutated CFTR were close to 0. Because RU values for F508del-CFTR expressing cells were
17 positive above 50ng and because CFTR is not the only glycosylated protein in cell lysates that
18 could bind to WGA, the CFTR antibody was injected during the dissociation phase. An
19 example of resulting sensorgram is given in Fig. 6C (left). When the quantitation was
20 performed, a medium amount of proteins was injected (500ng). It was observed that the
21 binding of the antibody on the protein extracts themselves injected over WGA was
22 significantly higher in proteins from the Wt-CFTR expressing cells. For the quantitation, the
23 base line was taken just before the injection of the antibody. From the experiments performed
24 with Hela cells, we concluded that it is possible using an immobilized anti-CFTR antibody to
25 detect the protein in whole cell lysates and that the use of a combination of antibody and
26 WGA permits to discriminate between fully glycosylated CFTR and F508del-CFTR.
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43 Because the amount of CFTR protein is higher in transfected cells than in cells from
44 patients, it is challenging to detect the protein in primary cell lines. Therefore, some
45 experiments were done with cells from CF patients (n=3) and compared with controls (n=3).
46 Due to the difficulty to obtain nasal cells from patients, to culture them and to obtain a
47 sufficient amount of proteins, only one sort of experiment was performed. 1 μ g of total
48 proteins was injected on the immobilized anti-CFTR antibody and 0.5 μ g of WGA was
49 injected during the dissociation phase (Fig. 7A). Proteins extracted from Hela cells transfected
50 with an empty plasmid were used as control. Each injection was performed twice. Once more,
51 a lower binding of CFTR on the antibody was observed for the proteins extracted of mutated
52 CFTR expressing cells. Proteins extracted from Hela cells transfected with the empty plasmid
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3 gave a medium association but dissociation comparable to the one of CF cells. The base line
4 was taken just before WGA injection to avoid the bias due to the binding on the antibody.
5 When WGA was injected, a lower binding on CF proteins was also observed. 20 seconds after
6 the beginning of the dissociation phase RU values were collected and used in statistical
7 analysis. The result is shown in Fig. 7B as a histogram in which RU values of non-transfected
8 Hela cells was given to 1 unit. Comparison of this control with the binding of WGA on
9 control protein indicated that nasal cell significantly express more CFTR than Hela cells. The
10 comparison of the results between proteins from control nasal cells and CF nasal cells showed
11 that WGA was significantly less bound on CF proteins and that the level of bound WGA was
12 identical to the one observed for empty Hela cells. We concluded that it is possible to detect
13 CFTR in proteins from primary cells and that the use of WGA permits to discriminate mature
14 CFTR from F508del-CFTR.
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23 **4. Discussion and conclusion**

24 The assessment of the expression of CFTR, as well as the assessment of its maturation
25 is of main importance in the CF field of research, at the basic science level but also when the
26 aim it to test correctors that could rescue F508del-CFTR from the ER. Knowing whether
27 CFTR is expressed in a given cell line is not sufficient. Indeed, it is fundamental to know
28 whether the fully glycosylated mature form is expressed do to the fact that solely this form
29 acts as a normal Cl⁻ channel. Experimental procedures are time consuming and poorly
30 specific, needing SDS-PAGE and immunoblotting with antibodies that are not always fully
31 specific. The glycosylation state of CFTR has to be tested by glycosidase digestions and
32 immunoblotting [12]. The aim of the present study was to provide an easy, rapid and
33 reproducible methodology to assess whether the CFTR protein is expressed in a complex
34 protein extract and whether it is mature or not. SPR is a very sensitive tool to detect and
35 quantify protein interactions without any labelling of pre-treatment [19]. Nevertheless, it is
36 often used to study the interactions of purified proteins. We and others previously showed that
37 the detection of a single protein with a low expression, in a complex sample such as serum, is
38 feasible using SPR, and we show here that it can also be used to detect CFTR in a complex
39 sample [23]. Nevertheless, two detections in a single protein injection were necessary. Anti-
40 CFTR antibody may be poorly specific, leading to false results or to over estimations.
41 Furthermore, they do not permit to distinguish the mature form of CFTR. To avoid this bias,
42 we decided to use WGA that specifically recognize the mature form of the protein [24]. Using
43 total proteins from cells, we had to face the fact that WGA recognize many proteins.
44 Therefore, the single use of an antibody or WGA was not sufficient to detect the mature form
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of CFTR. A sandwich method had to be used and we propose here that the immobilization of anti-CFTR antibody permits to assess the expression of the protein and that a subsequent injection of WGA permits to distinguish its mature form. Furthermore, using purified CFTR protein or a control protein extract permits the absolute or relative quantitation of CFTR in a complex sample. When compared to classical western blot, another advantage of SPR is the use of a very low amount of proteins. Whereas 50 to 100µg of total proteins are necessary for the detection of CFTR in cell lysates, here we show that using SPR few ng are sufficient. The proposed method is of interest for fundamental research aimed to rapidly find correctors as well as for research aimed to show the potential therapeutic use of a given compound in cells obtained from patients.

In conclusion, we propose SPR as an alternative method to detect and give a relative quantitation of CFTR in a complex protein sample, in a short time, despite a low expression of the protein. Furthermore, we propose, for the first time, that this technology can also be used to discriminate the mature form of CFTR from its immature state providing a less time consuming and a more simple method than western-blottings and enzymatic digestions.

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References

- [1] Kerem, B.S., Rommens, J.M., Buchana, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M. and Tsui, L.C. (1989). Identification of the cystic fibrosis gene: genetic analysis. *Science* 245, 1073-1080.
- [2] Riordan, J.R., Rommens, J.M., Kerem, B.S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L., Drumm, M.L., Lannuzzi, M.C., Collins, F.S. and Tsui, L.C. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245, 1066-1073.
- [3] Rommens, J.M., Lannuzzi, M.C., Kerem, B.S., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, J.R., Tsui, L.C. and Collins, F.S. (1989). Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* 245, 1059-1065.
- [4] Welsh MJ, Tsui LC, Boat TF and Beaudet AL. in: C.R. Scriver, A.L. Beaudet, W.S. Sly and Valle, D. In *The Metabolic and Molecular Bases of Inherited Disease* (7th edition., McGraw-Hill, New York), (1995), pp. 3799-3876.
- [5] Drumm ML, Pope HA, Cliff WH, Rommens JM, Marvin SA, Tsui L, Collins FS, Frizzel RA and Wilson JM. Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. *Cell* (1990) 62: 1227-1233.
- [6] Rich DP, Anderson MP, Gregory RJ, Cheng SH, Paul S, Jefferson DM, McCann JD, Klinger KW, Smith AE and Welsh MJ. Expression of the cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature* (1990) 347: 358-363.

- 1
2
3 [7] Szellas T and Nagel G. Apparent affinity of CFTR for ATP is increased by continuous
4 kinase activity. *FEBS Letters* (2003) 535: 141-146.
- 5 [8] Ward, C.L. and Kopito, R.R. (1994). Intracellular turnover of Cystic Fibrosis
6 Transmembrane Conductance Regulator. *J. Biol. Chem.* 269, 25710-18.
- 7 [9] Gregory, R.J., Cheng, S.H., Rich, D.P., Marshall, J., Paul, S., Hehir, K., Ostedgaard, L.,
8 Welsh, M.J. and Smith, A.E. (1990). Expression and characterization of the Cystic Fibrosis
9 Transmembrane Conductance Regulator. *Nature* 347, 382-386.
- 10 [10] Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan,
11 C.R. and Smith, A.E. (1990). Defective intracellular transport and processing of CFTR is the
12 molecular basis for most of cystic fibrosis. *Cell* 63, 827-834.
- 13 [11] Denning, G.M., Ostedgaard, L.S. and Welsh, M.J. (1992). Abnormal localization of
14 Cystic Fibrosis Transmembrane Conductance Regulator in primary cultures of cystic fibrosis
15 airway epithelia. *J. Cell. Biol.* 118, 551-559.
- 16 [12] Kartner, N., Augustinas, O., Jensen, T.J., Naismith, A.L. and Riordan, J.R. (1992).
17 Mislocalization of Δ F508 CFTR in cystic fibrosis sweat glands. *Nat. Genet.* 1, 321-327.
- 18 [13] Lukacs, G.L., Mohamed, A., Kartner, N., Chang, X.B., Riordan, J.R. and Grinstein, S.
19 (1994). Conformational maturation of CFTR but not its mutant counterpart (Delta F508)
20 occurs in the endoplasmic reticulum and requires ATP. *EMBO J.* 13, 6076-6086.
- 21 [14] Denning, G.M., Denning, G.M., Anderson, M.P., Amara, J.F., Marshall, J., Smith, A.E.,
22 and Welsh, M.J. (1992). Processing of mutant cystic fibrosis transmembrane conductance
23 regulator is temperature-sensitive. *Nature* 358, 761-764.
- 24 [15] Kopito, R.R. (1999). Biosynthesis and degradation of CFTR. *Physiol. Rev.* 79, S167-
25 S173.
- 26 [10] Du, K., Sharma, M., and Lukacs, G.L. (2005). The Δ F508 cystic fibrosis mutation
27 impairs domain-domain interactions and arrests post-translational folding of CFTR. *Nat.*
28 *Struct. Mol. Biol.* 12, 17-25.
- 29 [16] Younger, J.M., Ren, H.Y., Chen, L., Fan, C.Y., Fields, A., Patterson, C. and Cyr, D.M.
30 (2004). A foldable CFTR (Delta) F508 biogenic intermediate accumulates upon inhibition of
31 the Hsc70-CHIP E3 ubiquitin ligase. *J Cell Biol.* 167(6), 1075-85.
- 32 [17] Gilbert, A., Jadot, M., Leontieva, E. Wattiaux-De Coninck, S., Wattiaux, R. (1998). Δ
33 F508 CFTR localizes in the endoplasmic reticulum-Golgi intermediate compartment in cystic
34 fibrosis cells. *Exp. Cell. Res.* 242, 144-152.
- 35 [18] Gregory, R.J., Rich, D.P., Cheng, S.H., Souza, D.W., Paul, S., Manavalan, P., Anderson,
36 M.P., Welsh, M.J. and Smith, A.E. (1991). Maturation and function of Cystic Fibrosis
37 Transmembrane Conductance Regulator variants bearing mutations in putative nucleotide-
38 binding domains 1 and 2. *Mol. Cell. Biol.* 11, 3886-93.
- 39 [19] Place, J.F., Sutherland, R.M. and Dähne, C. (1985). Opto-electronic immunosensors: a
40 review of optical immunoassay at continuous surfaces. *Biosensors* 1(4):321-53.
- 41 [20] Pollock, N., Cant, N., Rimington, T. and Ford, R.C. (2014). Purification of the Cystic
42 Fibrosis Transmembrane Conductance Regulator Protein Expressed in *Saccharomyces*
43 *cerevisiae*. *J Vis Exp.* May 10(87), e51447, doi:10.3791/51447.
- 44 [21] Teng, L., Kerbirou, M., Taiya, M., Le Hir, S., Benz, N., Trouvé, P. and Férec C.
45 (2012). Proteomic Identification of Calumenin as a G551D - CFTR Associated Protein. *PLoS*
46 *One*, 7(6): e40173.
- 47 [22] Mosler, K., Coraux, C., Fragaki, K., Zahm, J.M., Bajolet, O., Bessaci-Kabouya, K.,
48 Puchelle, E., Abély, M. and Mauran, P. (2008). Feasibility of nasal epithelial brushing for the
49 study of airway epithelial functions in CF infants. *J Cyst Fibros.* 7(1), 44-53.
- 50 [23] Trouvé, P., Kerbirou, M., Le Hir, S., Benz, N. and Férec, C. (2012). Surface Plasmon
51 Resonance shows a gender difference in circulating annexin A5 in human. *Talanta* 93 219-
52 223
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56
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59
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2
3 [24] O'Riordan, C.R., Lachapelle, A.L., Marshall, J., Higgins, E.A., Cheng, S.H. (2000).
4 Characterization of the oligosaccharide structures associated with the cystic fibrosis
5 transmembrane conductance regulator. *Glycobiology*. 10(11):1225-33.
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8 9 **Legends to the figures**

10
11 **Figure 1. Binding of non mutated CFTR and F508del-CFTR proteins on the anti-CFTR**
12 **antibody. A.** Representative sensorgrams obtained when 10, 20, 40, 50ng of purified
13 proteins were injected over the anti-CFTR. Higher responses are observed for the non
14 mutated CFTR protein. BSA was used as a negative control. **B.** Curves representing the
15 response (RU) in function of the injected amount of proteins. Whereas the response is linear
16 for both proteins in the range of the injected quantity of proteins, a significant higher binding
17 is observed for the non mutated CFTR. R^2 is the coefficient of determination of the curves.
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20 **Figure 2. Binding of non mutated CFTR and F508del-CFTR proteins on the lectin**
21 **(WGA). A.** Representative sensorgrams obtained when 10, 20, 40, 50ng of purified proteins
22 were injected over the anti-CFTR. Higher responses are observed for the non mutated CFTR
23 protein. The response due to the injection of F508del-CFTR protein is positive but weak.
24 BSA was used as a negative control. **B.** Curves representing the response (UR) in function of
25 the injected amount of proteins. The response is linear for both proteins in the range of the
26 injected quantity of proteins and is significantly higher for the non mutated CFTR protein.
27 The slope of the curve obtained with F508del-CFTR protein is very low showing a weak
28 binding on the lectin. **C.** Curves representing the response (UR) in function of the injected
29 amount of proteins below 50ng in order to see the behaviour of the curves in Fig. 2B. It
30 shows that for very low concentrations a response was observed for F508del-CFTR with a
31 maximum reached sooner than non mutated CFTR, with no further increase of the response
32 above 10ng. Linear curves were drawn to calculate the indicated slopes of the curves before
33 the plateau phase and to show that this plateau phase is reached sooner with F508del-CFTR
34 protein.
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38 **Figure 3. Binding of WGA on non mutated CFTR and F508del-CFTR proteins when**
39 **they are bound on the anti-CFTR antibody. A.** Example of sensorgram showing the
40 binding of the purified proteins on the antibody and showing the following association of the
41 lectin. Higher amounts of bound proteins on the antibody as well as a higher amount of
42 bound WGA are observed for the non mutated CFTR. **B.** Plotted curves of the binding of
43 WGA on increasing amount of linked non mutated CFTR and F508del-CFTR proteins (left).
44 The response due to WGA binding is increasing accordingly to the amount of bound non
45 mutated CFTR on the antibody whereas the response due to WGA binding on F508del-
46 CFTR remains low (<2RU). The histogram (right) represents the statistical analysis of WGA
47 binding and shows significantly higher RU values for the non mutated CFTR than for
48 F508del-CFTR ($p < 0.0001$).
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51 **Figure 4. Binding of the anti-CFTR antibody on non mutated CFTR and F508del-**
52 **CFTR proteins when they are bound on WGA. A.** Example of sensorgram showing the
53 binding of the purified proteins on the lectin and showing the following association of the
54 antibody. Higher amounts of bound proteins on WGA as well as a higher amount of bound
55 antibody are observed for the non mutated CFTR. **B.** Plotted curves of the binding of the
56 anti-CFTR antibody on increasing amount of linked non mutated CFTR and F508del-CFTR
57 proteins onto WGA (left). The response due to the antibody binding is increasing accordingly
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3 to the amount of bound non mutated CFTR on WGA whereas the response with F508del-
4 CFTR remains low. The histogram (right) represents the statistical analysis. The antibody
5 binding is significantly higher on the non mutated CFTR than on F508del-CFTR ($p < 0.0001$).
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7 **Figure 5. Detection of Wt-CFTR and F508del-CFTR proteins in stably transfected HeLa**
8 **cells using first an anti-CFTR antibody and secondly WGA. A.** Example of sensorgram
9 showing the binding of the proteins extracted from HeLa cells expressing either Wt-CFTR or
10 F508del-CFTR on the antibody. Non-transfected cells and BSA were used as negative
11 controls. It can be observed that proteins from both cell types gave positive results when they
12 were injected over the anti-CFTR antibody. **B.** Plots of the RU values obtained when 0 to
13 2 μg of proteins were. The upper panel shows the whole curves indicating that above 100ng
14 of proteins a plateau phase is reached. The lower panel is the curve obtained with 0 to 100ng
15 of proteins, showing that for low amount ($< 50\text{ng}$) of proteins, there was no difference
16 between the extracts of Wt-CFTR and F508del-CFTR expressing cells. **C.** Example of
17 sensorgrams obtained when 0.5 μg WGA was injected during the dissociation phase of the
18 whole cell extracts (left). A higher signal was observed for the Wt-CFTR expressing cells.
19 The responses due to the injection of WGA were quantified and the results are shown as
20 histograms (right). The difference between the proteins from the two cell lines is significant
21 ($p < 0.001$), the binding of WGA being more important on the CFTR from Wt-CFTR
22 expressing HeLa cells.
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26 **Figure 6. Detection of Wt-CFTR and F508del-CFTR proteins in stably transfected HeLa**
27 **cells using first WGA and then the anti-CFTR antibody. A.** Example of sensorgram
28 showing the binding of the proteins extracted from HeLa cells expressing either Wt-CFTR or
29 F508del-CFTR on WGA. It can be observed that the responses obtained with proteins from
30 F508del-CFTR expressing were very low. **B.** RU values were plotted against the amount of
31 injected proteins (upper panel). Below 100ng of injected proteins, the RU values were close
32 to 0 for the proteins from F508del-CFTR expressing cells. Lower panel is a higher
33 magnification of the curves with proteins ranging from 0 to 50ng. The curves show that the
34 values of the protein extracts containing the mutated CFTR were indeed close to 0. **C.**
35 Example of sensorgrams obtained when anti-CFTR antibody was injected during the
36 dissociation phase of whole cell extracts (left). The responses due to the injection of WGA
37 were quantified and the results are shown as histograms (right; $p < 0.001$).
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41 **Figure 7. Detection of Wt-CFTR and F508del-CFTR in proteins obtained from control**
42 **and CF nasal cells. A.** Example of sensorgram showing the successive binding of proteins
43 from healthy controls and CF patients on the antibody and the binding of WGA on the
44 retained proteins. Proteins extracted from HeLa cells transfected with an empty plasmid were
45 used as control. A lower binding on the antibody was observed for the proteins extracted of
46 F508del-CFTR expressing cells. Proteins extracted from HeLa cells transfected with an empty
47 plasmid had a dissociation phase comparable to the one of CF cells. Injection of WGA
48 showed a lower binding on CF proteins than on control proteins. **B.** Histogram in which RU
49 values of non-transfected HeLa cells was given to 1 unit, showing that WGA was significantly
50 less bound on CF proteins.
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Figure 1

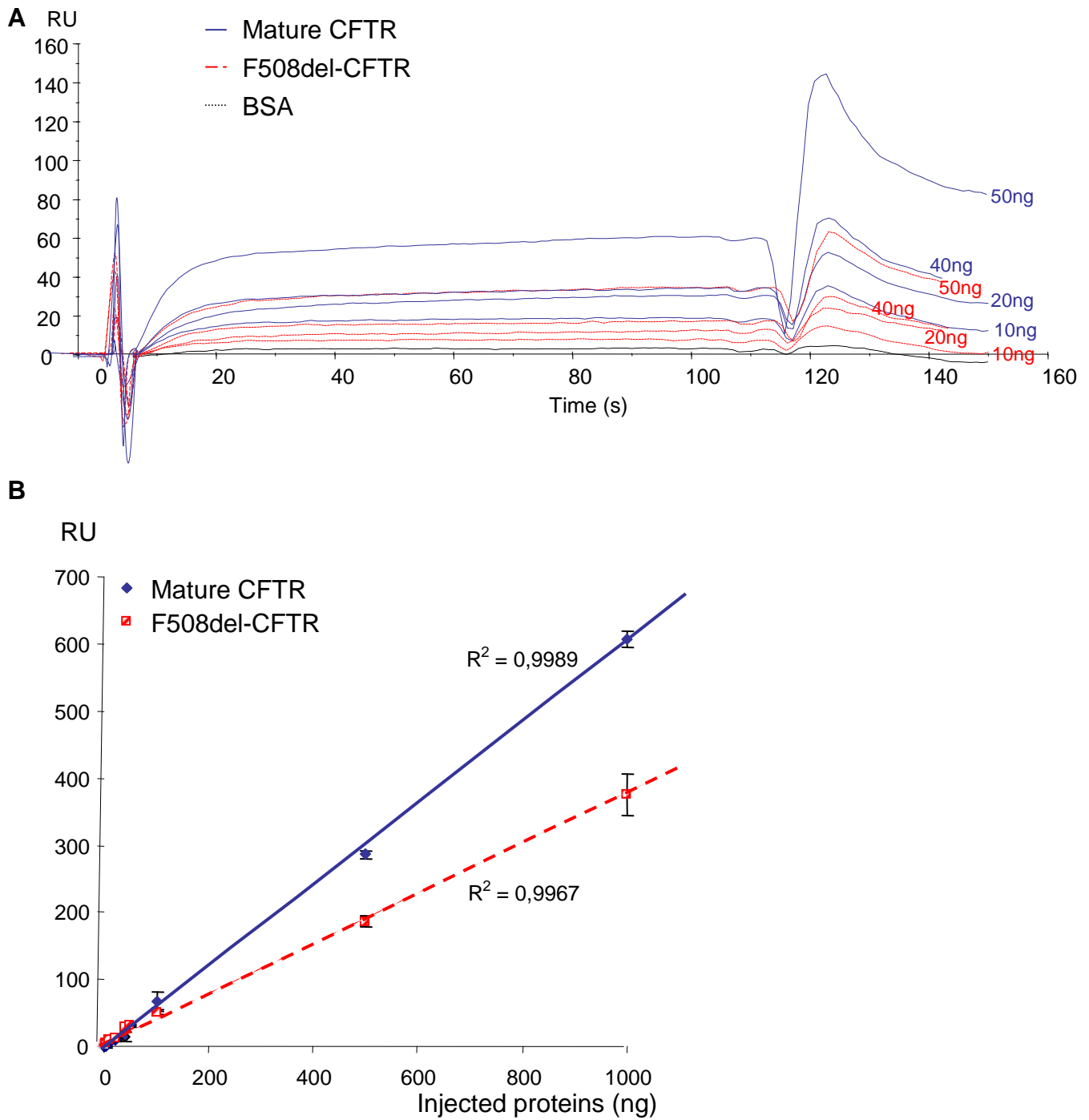


Figure 2

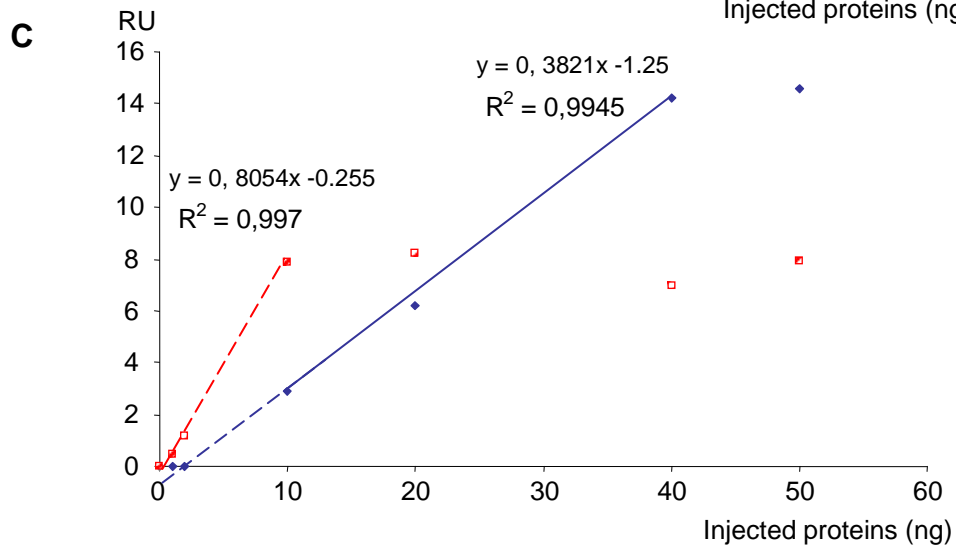
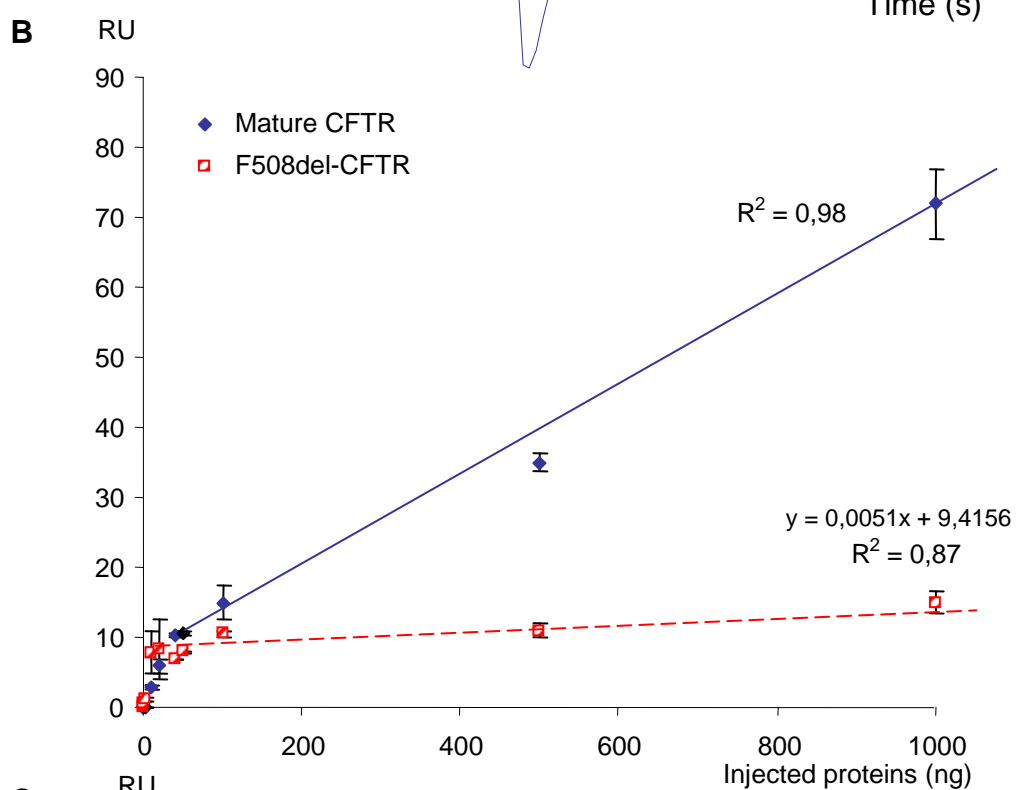
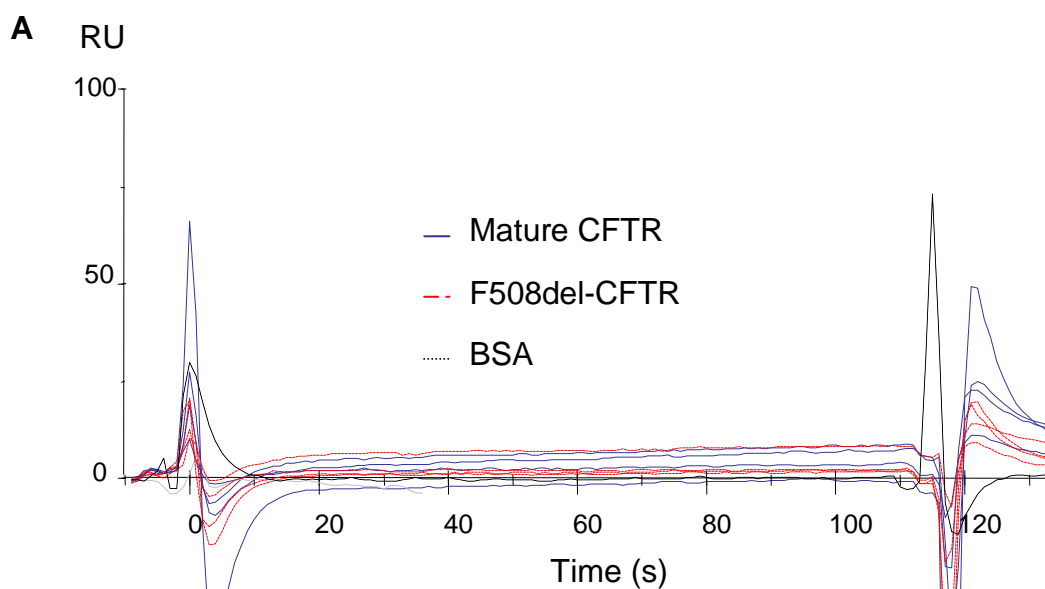


Figure 3

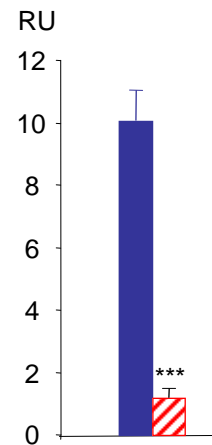
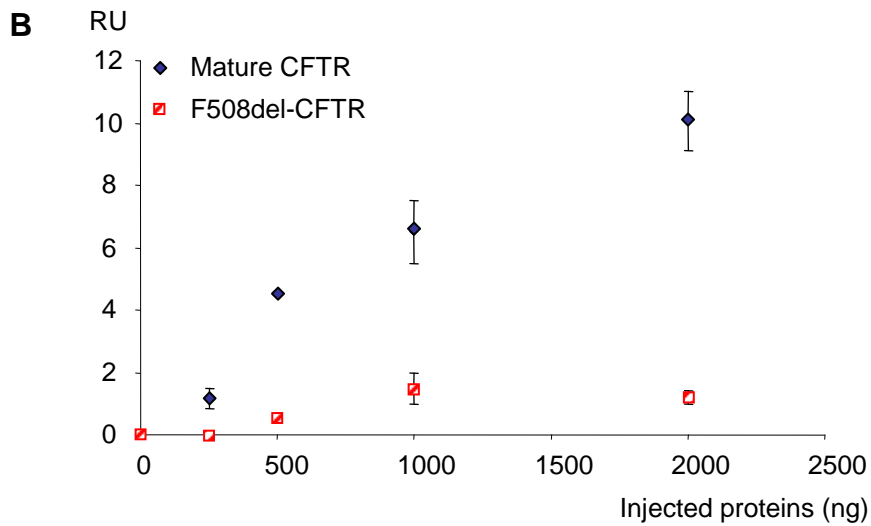
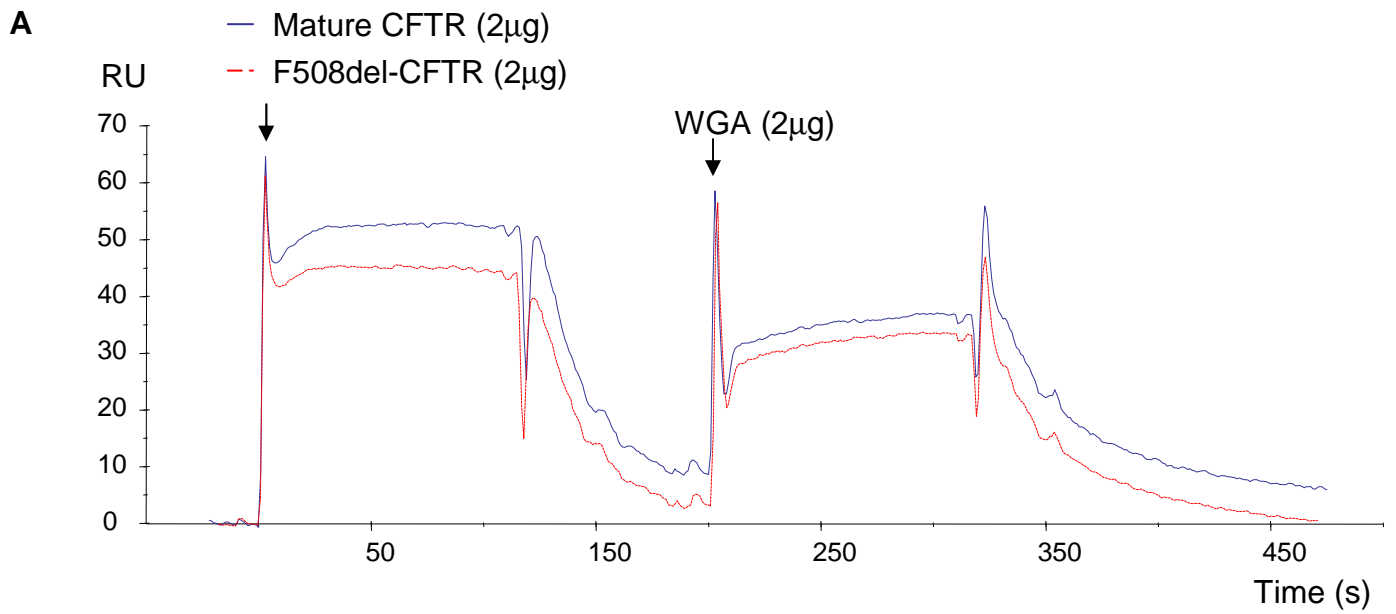


Figure 4

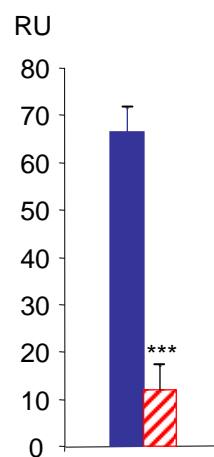
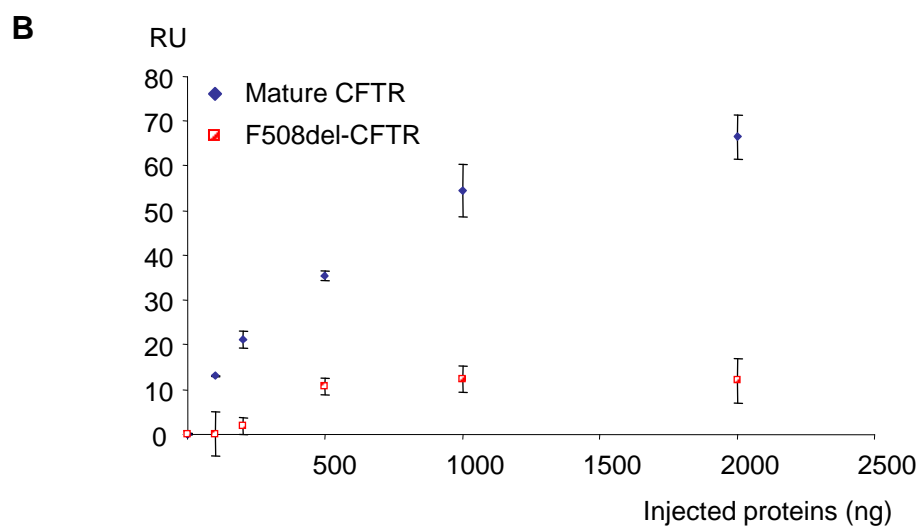
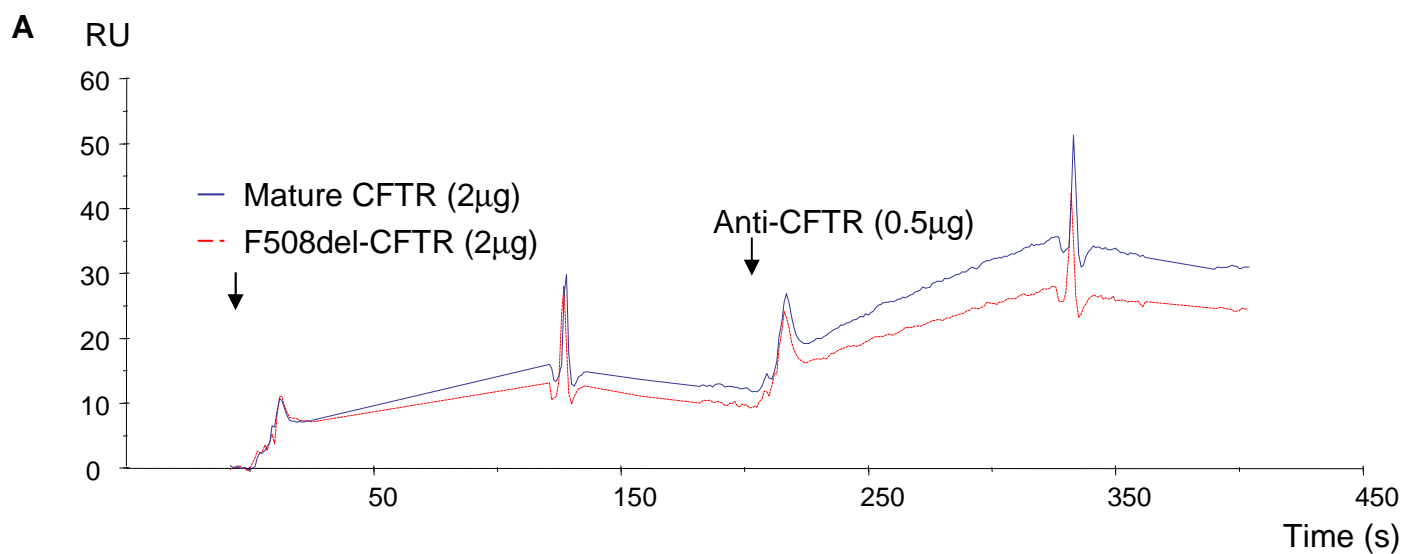


Figure 5

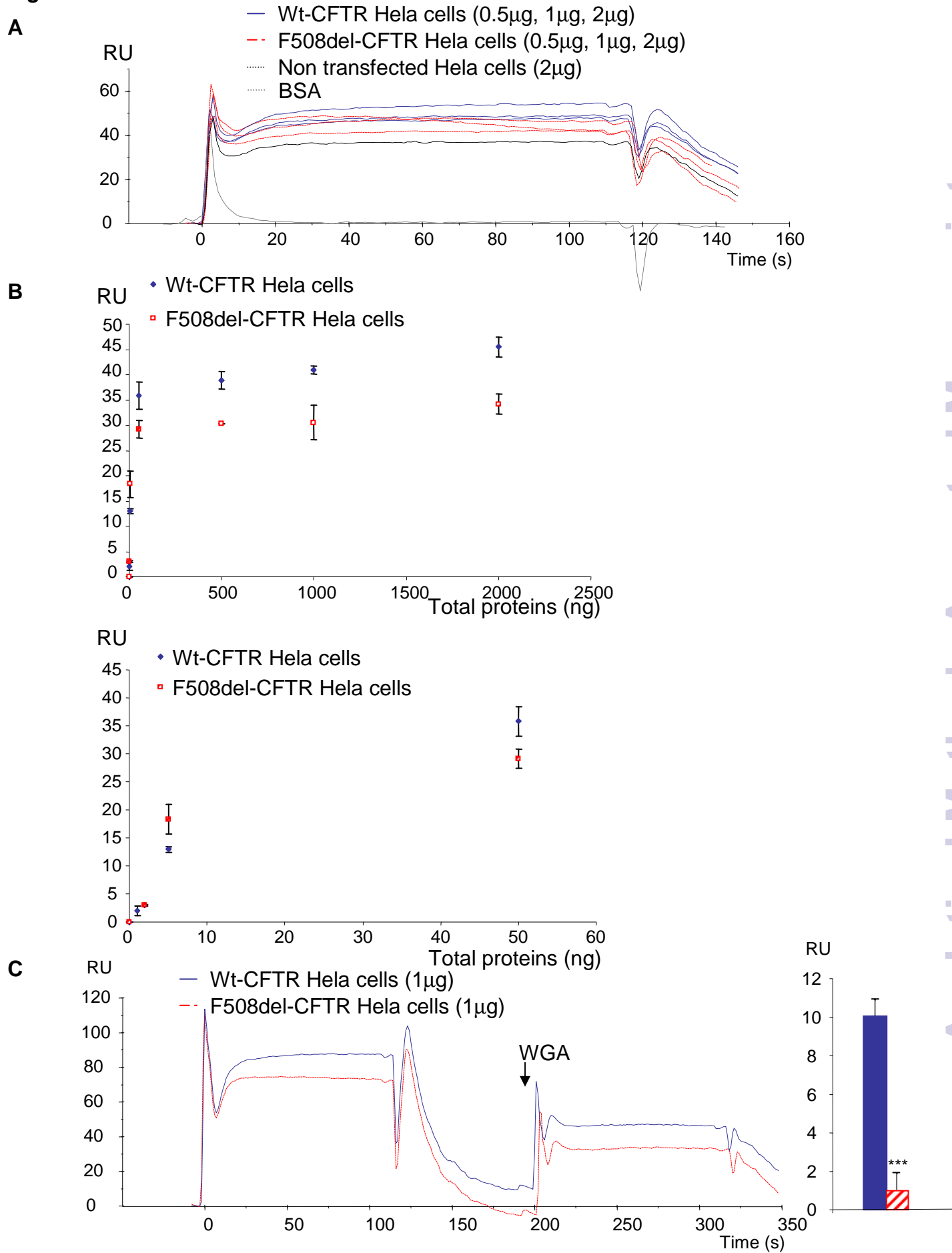


Figure 6

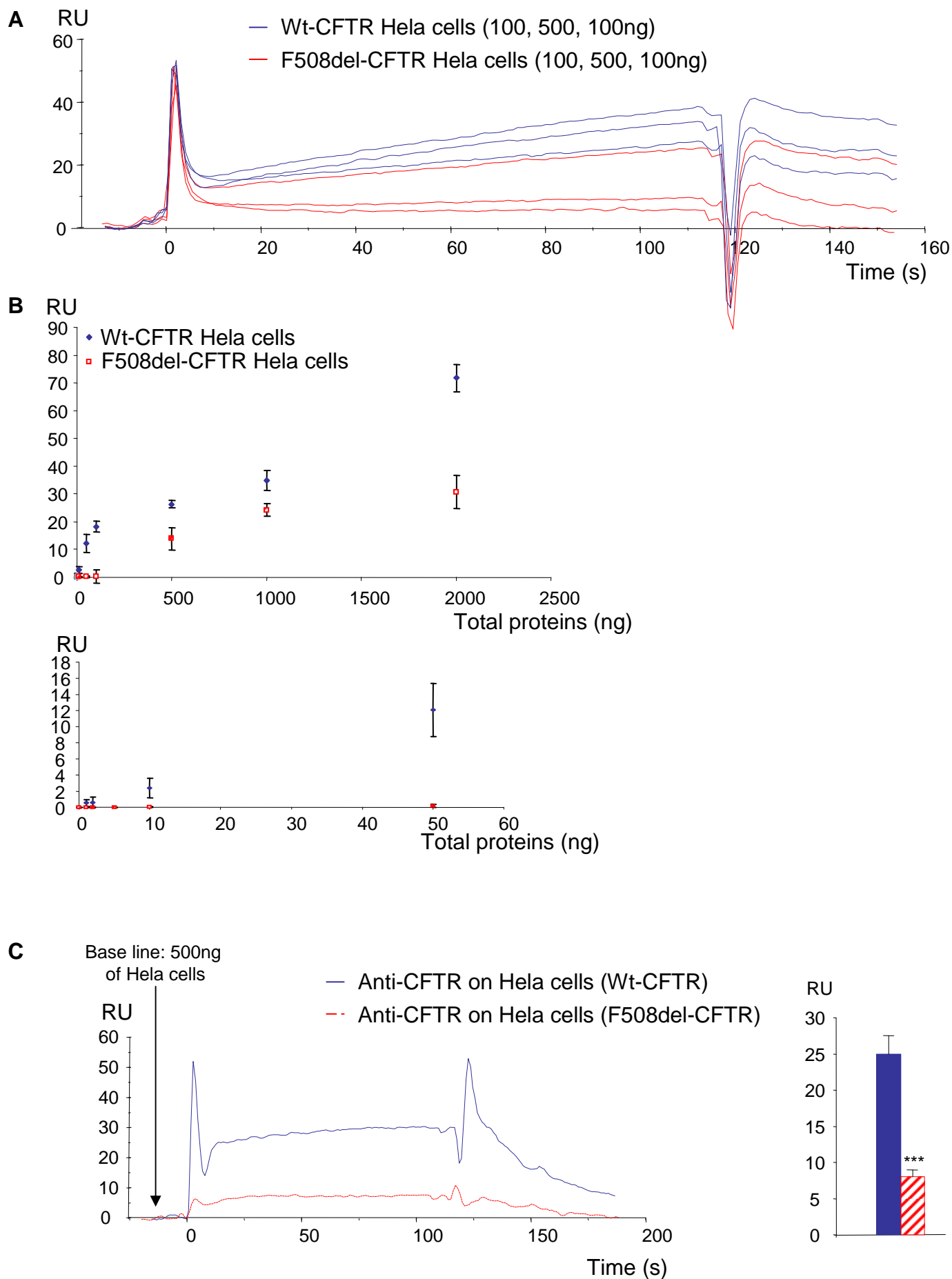


Figure 7

