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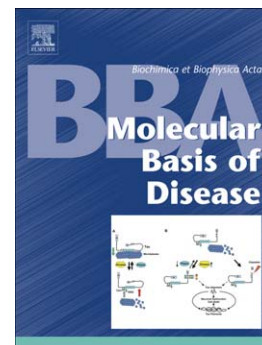
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## Functional analysis of F508del CFTR in native human colon

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**Abstract**

The major cystic fibrosis mutation F508del has been classified by experiments in animal and cell culture models as a temperature-sensitive mutant defective in protein folding, processing and trafficking, but literature data on F508del CFTR maturation and function in human tissue are inconsistent. In the present study the molecular pathology of F508del CFTR was characterized in freshly excised rectal mucosa by bioelectric measurement of the basic defect and CFTR protein analysis by metabolic labelling or immunoblot. The majority of investigated F508del homozygous subjects expressed low amounts of complex-glycosylated mature F508del CFTR and low residual F508del CFTR-mediated chloride secretory activity in the rectal mucosa. The finding that some F508del CFTR escapes the ER quality control in vivo substantiates the hope that the defective processing and trafficking of F508del CFTR can be corrected by pharmacological agents.

**Keywords:** cystic fibrosis; F508del CFTR protein analysis; intestinal current measurement; rectal biopsy

Abbreviations:

CF, cystic fibrosis

CFTR, cystic fibrosis transmembrane conductance regulator

Endo H, endo- $\beta$ -N-acetylglucosaminidase H

ER, endoplasmatic reticulum

ICM, intestinal current measurement

NBD, nucleotide binding domain

SI, sucrase - isomaltase

## 1. Introduction

Cystic fibrosis (CF) is a generalized disorder of exocrine epithelia that is caused by mutations in the *CFTR* gene (OMIM 219700) [1]. The single 3-bp deletion F508del is present on at least one allele in more than 90% of CF patients [2] thus making it the most common disease-causing lesion in autosomal recessive traits of Caucasian populations.

Phenylalanine 508 resides on the surface of the first nucleotide binding domain (NBD1) of CFTR [3]. Deletion of F508 exerts only minor local effects on NBD1 structure around position 508 [3, 4], but it precludes the proper assembly between the cytoplasmic and membrane-integrated domains of CFTR [5]. In vitro most of the newly synthesized F508del CFTR fails to adopt a wild-type fold in the ER [3] and is targeted for ER-associated degradation [6]. Chaperone-assisted modulation of the folding of the nascent protein [7] or biosynthesis at temperatures below 30 °C [8] can rescue F508del CFTR. However, any F508del CFTR protein that reaches the cell surface remains thermally unstable, capable of only partial chloride channel activity [9], and is rapidly endocytosed and finally delivered to the lysosome for degradation [10]. In summary, F508del CFTR has been classified as a trafficking- and processing-deficient mutation [11].

Concurrent with this classification inferred from cellular models, only mislocalized F508del CFTR protein was immunodetectable in F508del homozygotes' sweat glands [12, 13]. Literature reports on the F508del CFTR phenotype in human airways [13-17] and human intestine [13, 18, 19], however, are inconsistent. Our group has described the presence of the mature complex-glycosylated F508del CFTR on immunoblots from intestinal tissue [13] and residual F508del CFTR-mediated chloride conductance in some F508del homozygous intestinal epithelia [18]. These findings were challenged by a subsequent study which applied a new set of anti-CFTR antibodies [19]. No F508del CFTR mature protein or function could be detected in rectal mucosa [19].

Considering the conflicting outcome of our initial and the more recent study we decided to re-investigate the patients' F508del CFTR phenotype. Clarification of the molecular pathology of F508del CFTR in human tissue is not only of academic interest. In the light of the discovery of synthetic small molecules [20] that either reinforce residual CFTR activity ('potentiators') or normalize processing and trafficking of CFTR to the apical membrane ('correctors') it is critically important to know whether or not some properly processed F508del CFTR protein with residual activity is present in the affected individuals.

In the present study the molecular pathology of F508del CFTR was characterized in freshly excised rectal mucosa by bioelectric measurement of the basic defect followed by CFTR protein analysis. The ex vivo analysis of rectal suction biopsies revealed that the majority of investigated F508del homozygous individuals expressed low amounts of complex-glycosylated mature F508del CFTR and residual F508del CFTR-mediated chloride secretory activity in the rectal mucosa.

## 2. Materials and Methods

### 2.1. Subjects and volunteers

Twenty F508del homozygous (Supplemental Table S1), one R553X homozygous and one CFTRdele2,3 (21 kb) homozygous CF-subjects were examined. Their clinical status was determined by weight predicted for height (wfh) % (representative for the nutritional status) and FEVPERC (representative for the pulmonary status) [18]. Biopsies of non-CF healthy volunteers (n = 30, age of 19 – 43 years) were characterized by ICM together with biochemical CFTR analysis. The study (no. 2771) was approved by the Ethics Committee of Hanover Medical School. All participants of the study and/or their parental guides provided written informed consent.

### 2.2. Rectal suction biopsies and lung epithelium specimens

Two to four rectal biopsies were collected with a suction biopsy device (Model SBT-100, Trewavis Surgical, Bayswater, Australia) from each subject (see the typical histology of a non-CF biopsy in Supplemental Fig. S1). After intestinal current measurement (ICM) the specimens were immediately processed for immunohistochemistry or biochemical analysis. Histology was assessed in hematoxylin-eosin stained 4 µm sections of paraffin-embedded biopsies that had been fixed with formaldehyde.

Native CF lung epithelium specimens were obtained from freshly explanted CF lungs on the occasion of lung transplantation and snap frozen in liquid nitrogen. Non-CF lung epithelium specimens were analyzed from parts of non-CF lung explants that underwent structural control by histology, demonstrating regular airway epithelium and submucosal glands.

### 2.3. ICM

The electrogenic transport of ions across the intestinal epithelium was measured as short circuit current ( $I_{sc}$ ) by ICM following our previously published protocol and evaluation algorithm [18, 21]. Biopsies of the rectal mucosa were mounted into an adapted micro-Ussing chamber with an aperture of 1.13 mm<sup>2</sup>. Basal transepithelial resistance of the tissue was determined by measuring voltage response to pulse currents of 1  $\mu$ A, and basal  $I_{sc}$  was calculated from the basal and the open-circuit transepithelial resistance (Supplemental Table S2).

### 2.4. Antibodies

The following anti-CFTR Abs were used: polyclonal R453 [22] and R16 against the N-terminus (aa 1-14, R16/34) and C-terminus (aa 1466-1480, R16/33), polyclonal R40 [19] against C-terminus (aa 1465-1488), polyclonal PAC865 [13] against NBD2 (aa 1377-1409), mAb 596 [19] against NBD2 (aa 1204-1211), mAb 570 [19] against R-domain (aa 731-742), mAb L12B4 [12] against NBD1 (aa 386-412, Chemicon) and mAb M3A7 [12] against NBD2 (aa 1370-1380, Chemicon). All antibody lots were verified to recognize the B- and C-bands of CFTR in T84 cells. For more information about the characteristics of the polyclonal anti-CFTR antibodies the reader is referred to Supplemental Table S3.

### 2.5. CFTR immunoblot analysis

The freshly excised biopsy specimens (n = 2 – 4) were homogenized in the presence of 10 mM iodoacetamide, 20 mM PMSF, 20  $\mu$ g/ml pepstatin and antipain, 100  $\mu$ g/ml leupeptin and aprotinin and 500  $\mu$ g/ml soybean trypsin-inhibitor in Tris-buffer (20 mM Tris/HCl, 150 mM NaCl, pH 8). The lysis started by incubation with 0.03 % SDS for 30 min, followed by 1 % (v/v) Triton X-100 and 0.5 % (w/v) sodium deoxycholate for 30 min. After centrifugation (16,000 $\times$ g, 4°C, 20 min) the supernatant was incubated with protein A-sepharose (PAS, GE



Healthcare) or with the specific pre-immune serum and PAS for 60 min. Immunoprecipitation (IP) was carried out overnight with the polyclonal antibodies R40 (1:50) and R453/R16 (1:50) or R40 (1:50) plus R16/33 (1:40) and R16/34 (1:67) in the presence of PAS (GE Healthcare) and protein G-agarose (Santa Cruz). All pellets were washed several times with 1) PBS with 0.5 % (v/v) Triton X-100 and 0.05 % (w/v) sodium deoxycholate and 2) 125 mM Tris, 500 mM NaCl, 10 mM EDTA, 0.5 % (v/v) Triton X-100, pH 8. CFTR immunoreactive bands were detected on 5 % SDS-PAGE separated PVDF-membranes with mAbs 570 and 596 (1:500) in 0.2 % I-Block (Tropix, Applied Biosystems) in 0.05% Tween-TBS (T-TBS) and pre-adsorbed anti-mouse-IgG-HRP from donkey (1:300000, abcam) in 0.2 % I-Block in T-TBS incubating with ECL Advance (GE Healthcare) for 20 seconds. Exposure times were chosen between 20 sec and up to 20 min as time series for each sample. The non-CF samples were exposed between 5 sec and up to 5 min. As markers for calibration of the CFTR B- and C-bands in rectal biopsies the B- and C-bands of T84 immunoprecipitates separated on the same gel and the protein marker Precision Plus Protein Standards All Blue (10 -250 kDa, BIO-RAD) were used. The amount of protein (experimental error  $\pm$  11 %) was determined by the Bradford assay [23] and the CFTR immunoreactive signal was quantified by densitometry of a time series of Hyperfilms ECL (GE Healthcare) exposed to ECL Advance – covered immunoblot. For digestion with endo- $\beta$ - N- acetylglucosaminidase H (Endo H, Roche) immunoprecipitated proteins were eluted in 0.5 % SDS and 1 % Mercaptoethanol at 37 °C for 1 h. The treatment with Endo H was performed as described by Naim et al. [24].

### *2.6. Metabolic labelling*

After ICM two rectal biopsies were placed on a stainless steel grid and labelled biosynthetically with 15.4 MBq [<sup>35</sup>S]cysteine and 18.5 MBq [<sup>35</sup>S]methionine (GE Healthcare) in methionine and cysteine deficient D-MEM medium (Invitrogen) supplemented with streptomycin and penicillin (Invitrogen) following the protocol that was initially established

for the analysis of lactate-phlorizin hydrolase [24, 25] and more recently adapted for the analysis of CFTR [22]. The biopsies were incubated for 15 h at 37 °C, but the controls of non-CF in the lane a and c in Fig. 7 only for 5 hours and in lane b for 16 h [22]. Lysis, IP and SDS-PAGE were performed as described in the immunoblot section. The antibodies used for the IP are shown in the heading of Fig. 7 and 8. The vacuum-dried gels were exposed to Biomax MR1 films (Kodak). For calibration of the CFTR <sup>35</sup>S-labelled signals the labelled signals of the control protein sucrase-isomaltase (SI) [22] and the Prestained Molecular Weight Marker ( mol wt 26.6-180 kDa, Sigma) were used. However, the molecular weights of the 7 major bands varied between lots. The marker protein alpha 2-macroglobulin from human plasma as highest standard band varied between 167.7 to 187.3 kDa.

## Results

### *3.1 Immunodetection of CFTR in rectal biopsies: set-up of the protocol*

CFTR protein analysis in human specimens is critically dependent on a sufficient specificity and sensitivity of the applied immunochemical tools. This work employed a panel of own polyclonal peptide anti-CFTR antibodies (Fig. 1) and new generation high affinity monoclonal anti-CFTR antibodies the latter of which had been developed in the course of the recent studies by our peers on F508del homozygotes [19].

In our standard protocol rectal suction biopsies were lysed, the lysate was immunoprecipitated with anti-CFTR Abs, and CFTR then visualized by anti-CFTR mAb on the immunoblot of the PAGE-separated IP. A strong band C of the mature complex-glycosylated CFTR and a faint band B of the immature core-glycosylated CFTR were regularly detected in rectal biopsies collected from non-CF control subjects (Fig. 1A, lane c; Fig. 1C). Neither the pre-immune sera nor the secondary Ab produced any non-specific by-bands in the sensitive mol. wt. range for CFTR detection of above 100 kDa (Fig. 1A, lanes a, b). The various polyclonal anti-CFTR Abs which were used for immunoprecipitation in the

course of the study resulted in comparable patterns of immunoreactive CFTR signals on blots (Fig. 1C, lanes a, b vs. c, d). Incubation with EndoH led to the expected shift of the faint band B to the non-glycosylated band A (Fig. 1B) which verified that band B represented the mannose-rich ER isoform of CFTR. Immunodetection of CFTR was possible in fresh and snap-frozen biopsies, however, the same amount of protein lysate derived from frozen and fresh biopsies revealed a fainter CFTR signal in the former (data not shown). By strictly adhering to the upper limit of allotted biopsies per subject approved by the ethics committee, F508del CFTR protein analysis was reliably feasible in our hands with fresh, but not with frozen samples.

Figure 2 compares the CFTR immunoreactive signals in samples from lungs and distal intestine. The mature glycoform C differed in its glycosylation pattern between the two tissues and the normalized CFTR signal was about sevenfold lower in non-CF lung specimens ( $n = 7$ ) than in non-CF rectal biopsies ( $n = 30$ ). F508del CFTR immunoreactive signals from lung explants were either not detected in immunoblots ( $n = 2$ ) or marginally above the sensitivity threshold of detection ( $n = 5$ ). No complex-glycosylated F508del CFTR was detectable.

### *3.2 Study population*

Supplemental Table S1 compiles the clinical status of the 20 F508del homozygous subjects (median age: 13.8; range 7.3 – 36.8 years) at the day of investigation when they provided rectal biopsies for the characterization of their mutant CFTR phenotype. The recruited subjects were mildly or moderately affected by CF disease, only five of the 20 subjects were more compromised in their lung function than their average CF peer of the same age and gender (Supplemental Table S1). The eldest female (no. 3) and male participants (no. 5) were the most mildly affected F508del homozygous subjects at the CF clinic Hannover who had never been hospitalized because of CF-related symptoms.

### 3.3 ICM of F508del homozygous rectal mucosa

Of the 20 F508del CFTR homozygous subjects who provided rectal suction biopsies for CFTR protein analyses, the ICM revealed low anion secretory activity in the colonic epithelium of 18 individuals. Carbachol typically evoked a small residual anion secretory response of 0.1 – 3.8  $\mu\text{A}/\text{cm}^2$  in the F508del homozygous rectal mucosa, whereas samples of non-CF individuals exhibited mean responses of 30  $\mu\text{A}/\text{cm}^2$  (Fig. 3, Supplemental Table S2). The ICM tracing of the biopsy taken from CF subject 9 with close-to-normal response to cAMP/forskolin was a spectacular outlier (Fig. 4). All other ICM recordings from F508del homozygotes revealed responses in the typical CF range (Supplemental Table S2) [21, 26, 27]. Both DIDS-insensitive (i.e., F508del CFTR) and DIDS-sensitive alternative anion conductances [21] contributed to variable extent to the anion secretory activity of F508del homozygous rectal mucosa (Supp. Table S2), whereas in non-CF tissue virtually all anion secretion was attributed to CFTR as indicated by matching responses to carbachol and histamine (Fig. 3A). The two homozygotes for the nonsense mutation R553X or the large out-of-frame deletion CFTRdele2,3(21kb) in the *CFTR* gene were expected to express no functional CFTR. These CF disease controls showed indeed full-blown CFTR loss-of-function phenotypes in the ICM with no net anion secretory responses to carbachol, cAMP and histamine (Fig. 3B).

The ICM protocol adhered to the standardized procedure [21, 28] established by Veeze et al. [29, 30]. Data of this study could thus be compared with all previous recordings with the same ICM setup, registration mode and protocol at the CF centres in Rotterdam and Hannover on 269 non-CF and 301 CF subjects [27]. All F508del homozygotes investigated in this study but subject 9 had a cumulative value of the responses ( $\Delta I_{\text{sc carbachol}} + \Delta I_{\text{sc cAMP/forskolin}} + \Delta I_{\text{sc histamine}}$ ; median: 5.5, range: -2.0 to 13.2, outlier: 46.8  $\mu\text{A}/\text{cm}^2$ ) within the typical CF range

usually far below the cut off at  $34 \mu\text{A}/\text{cm}^2$  that is the best diagnostic ICM parameter to differentiate CF from non-CF [27].

### *3.4 CFTR protein analysis of F508del homozygous rectal mucosa*

CFTR protein analysis of rectal biopsies was performed immediately after ICM by the two complementary techniques of either immunoblot or metabolic labelling that identify steady state levels or newly synthesized protein, respectively. Since rectal biopsies were processed individually by subject, the work-up steps strictly adhered to the standardized protocols described above in order to minimize any technical in-between sample variation of the detected CFTR signals.

Biopsies collected from non-CF subjects showed a strong complex-glycosylated C-band and a weak mannose-rich B-band of CFTR in the immunoblot (Fig. 5, lane a). F508del CFTR-immunoreactive signals of rectal biopsies of F508del homozygous subjects were in the range of the B-band of non-CF controls (Fig. 6). The F508del CFTR B and C isoforms were detected in highly variable absolute and relative amounts in the rectal mucosa of the individual CF donors (Fig. 5, lanes b-f; Fig. 6, Table 1, Supplemental Figure S2). The complex-glycosylated isoform was visible in ten and the mannose-rich isoform in all thirteen F508del homozygous specimens analyzed by immunoblot. The normalized densitometric signal of the F508del CFTR glycoform C varied between 0 and 14% (median: 3%) of the signal of wild type band C (Fig. 6, Table 1), the ratio F508del C / F508del B varied between 0 and 40 (median 0.8) (Table 1). The expression levels of B-, C-isoform or total F508del CFTR did not correlate with the signal intensity of the DIDS-insensitive chloride secretory response in ICM (Supplemental Table S2). In other words, the electrophysiological signature of a F508del homozygous biopsy in the ICM was not associated with its profile of F508del CFTR protein isoforms.

Metabolic labelling of rectal biopsies with [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine and subsequent IP with a cocktail of anti-CFTR antibodies led to the appearance of bands that were consistent with the expected shape and size of core- and complex glycosylated CFTR isoforms (Fig. 7). Based on established protocols for the metabolic labelling of intestinal biopsies [24, 25], we had adapted and optimized the procedure for the analysis of CFTR [22]. Thanks to this previous work all experiments of this study from non-CF (n = 8) and F508del homozygous donors (n = 7) yielded interpretable data. To facilitate the assignment of bands in the autoradiographs, lysed biopsies were first immunoprecipitated with the anti-CFTR antibodies and subsequently with polyclonal antibodies raised against the marker protein sucrase-isomaltase (SI) [22]. These controls helped to assign bands to CFTR isoforms B and C in the autoradiographs of anti-CFTR IPs (Fig. 7B). The autoradiographs showed a further diffuse band of higher mobility than band C, but lower than that of band B (Figures 7 and 8). Digestion of the IPs with EndoH deglycosylated this band and band B and shifted their position to that of the deglycosylated CFTR A band (Fig. 8A). Hence both proteins are core-glycosylated. The cocktail of our anti-CFTR antibodies utilized for IPs was known to cross-react with one CFTR-unrelated protein in the molecular weight range of CFTR isoforms. This non-specific by-band, however, was sharp and thus distinct from the diffuse EndoH-sensitive band (see Fig. 8A, lane b). Hence we concluded that this diffuse band should represent a further isoform of CFTR that provisionally was named band B\* because of its sensitivity to EndoH. The diffuse band B\* has already been seen before in pulse-chase and immunoprecipitation experiments [31] when CFTR-transfected baby hamster kidney cells were treated with the tyrosine kinase inhibitor tyrphostin 47 that enhances CFTR-mediated transepithelial chloride transport [32]. Since this band B\* has yet only been observed by metabolic labelling, it probably is an intermediate of post-ER processing of CFTR.

Metabolic labelling of rectal biopsies of non-CF (n = 8) and CF origin (n = 7) revealed no significant differences in the band patterns of autoradiographs of PAGE-separated anti-

CFTR IPs (Fig. 7A). The mature CFTR isoform was present in all investigated F508del homozygous samples. Thus the complex glycosylated F508del CFTR isoform was detected in wild type amounts in the fraction of newly synthesized F508del CFTR molecules, but the total amount of the mature C F508del CFTR glycoform was one to several orders of magnitude lower than that of complex glycosylated CFTR in non-CF rectal mucosa (Fig. 6; see also Fig. 8B for F508del CFTR protein analysis by immunoblot and metabolic labelling on fresh rectal samples from the same subject).

### Discussion

F508del CFTR is the most common mutant amongst all severe monogenic diseases of Caucasian populations and thus has received substantial attention to resolve its molecular pathology [3-13, 18, 19]. Investigations in cell culture models have allowed to classify the F508del CFTR mutant as a temperature-sensitive folding-trafficking defect [3-11] that retains residual chloride secretory activity if delivered to the plasma membrane [9]. Hence the conflicting outcome of ex vivo studies on human F508del CFTR homozygous distal intestine [13, 18, 19] may prima facie be unexpected.

Mall and colleagues measured chloride secretory activities in rectal biopsies from 12 F508del homozygous individuals [19]. Their ICM protocol [33] differs in numerous aspects such as buffer constituents, sequence of secretagogues and mode of recording from the procedure applied in this study [18, 21, 29, 30]. Cholinergic activation following IBMX(3-isobutyl-1-methylxanthine) /forskolin led to a maximal apparent chloride secretory response of 3.3  $\mu\text{A}/\text{cm}^2$ . This value reported by Mall and colleagues [19] is close to that of the DIDS-insensitive histamine-evoked chloride secretory response seen in this study. Thus both protocols unravelled minute residual chloride secretory activity of F508del CFTR. In other words, the two distinct protocols uncovered – at least semiquantitatively – similar electrophysiological phenotypes of F508del CFTR rectal biopsies in the ICM. A major

discrepancy between the outcome of the two studies, however, resides in the F508del CFTR immunoblot data.

Mall and colleagues [19] cryopreserved the rectal biopsy specimens after ICM. Subsequent CFTR protein analysis revealed only the immature band B isoform for samples from F508del homozygous individuals. Samples from normal individuals and heterozygous F508del gene carriers exhibited both the complex-glycosylated and the core-glycosylated CFTR isoforms on blots [19]. In contrast, this study demonstrated complex-glycosylated CFTR in more than 80% of investigated F508del homozygous and in all non-CF rectal biopsies on blots. Metabolic labelling of newly synthesized protein [22] revealed the mature isoform in all analyzed F508del homozygous samples. We took meticulous care that differences in the immunochemical detection of CFTR could not account for discordant primary data. Hence the immunochemical analysis of F508del CFTR was performed to large extent with the same set of mAbs as used by Mall and colleagues [19]. The major difference between the two studies was the F508del CFTR protein analysis of either frozen or freshly excised biopsies for the detection of mutant CFTR. In our hands the CFTR immunoreactive signal of non-CF biopsies became weaker by freezing and thawing. This procedure had a stronger impact on the global transcriptome than the interindividual differences in gene expression (data not shown).

Correspondingly we assume that the cryopreserved material analyzed by Mall and colleagues [19] had probably lost the labile F508del CFTR signal below the limit of detection due to the modes of sampling, storage and handling prior to CFTR immunochemical analysis. However, if the freshly excised sample is rapidly processed with an optimized protocol, the C-band of F508del CFTR is immunodetectable on blots. In other words, F508del CFTR is a labile protein in the native environment in accordance with the data gained in model systems, but nevertheless complex glycosylated F508del CFTR exists in most CF subjects in amounts of 0.1 – 14% of wild type CFTR in non-CF subjects (Fig. 6, Table 1). This fundamental



finding on the fate of F508del CFTR in F508del homozygous intestinal epithelium substantiates the hope that corrector molecules [20] such as VX-809 currently tested in clinical trials may increase the efficacy of the rescue so that clinically meaningful amounts of core-glycosylated F508del CFTR are stably converted to the mature glycoform. The combined administration of a corrector together with a potentiator of channel activity such as VX-770 [34] could then ameliorate the basic defect of F508del CFTR.

Our study on the combined ICM and F508del CFTR protein analysis of the same specimen had the unanticipated outcome that the anion secretory activity in the ICM was not associated with the pattern of F508del CFTR glycoforms in the immunoblot. The responses to carbachol, forskolin/cAMP and histamine in the ICM have been interpreted to represent chloride secretory responses with CFTR being the key player [18, 21, 29, 30]. Consistent with this interpretation, the cumulative value of the responses  $\Delta I_{sc \text{ carbachol}}$ ,  $\Delta I_{sc \text{ cAMP/forskolin}}$  and  $\Delta I_{sc \text{ histamine}}$  has empirically been found to represent the best ICM parameter to differentiate CF from non-CF [27].

Recent work, however, has demonstrated that a component of the carbachol-activated current in mouse colon is carried by bicarbonate through bestrophin Best2 channels [35]. Best2 is expressed at similarly high levels in human F508del homozygous and non-CF rectal biopsies [36]. Thus the carbachol-secretory response is probably composed of more than two currents, i.e. first, CFTR-mediated chloride and bicarbonate secretion, second, non-CFTR-mediated chloride secretion and third, Best2-mediated bicarbonate secretion. The secretory response to cAMP/forskolin is also not exclusively mediated by CFTR in the intestine. An alternative signalling pathway via the Epac-Rap-PLC- $[Ca^{2+}]_i$  cascade stimulates chloride secretion through a yet unknown chloride channel [37]. This Epac pathway could reasonably explain the anomalously high response of the F508del homozygous subject 9 to cAMP/forskolin (see Fig. 4). The lack of specificity of the carbachol- and the cAMP/forskolin – evoked secretory responses was already known when the ICM protocol was designed.

Hence the inhibitor DIDS is added prior to stimulation with the secretagogue histamine to block almost all chloride conductances but CFTR [21, 29, 30]. DIDS is not a specific inhibitor of CFTR, but the chosen high concentration of 0.2 mM is still the best tool to virtually inhibit all chloride conductance in the ICM but CFTR. Alternative drugs to dissect the CFTR-mediated signal from other conductances such as potentiators like genistein or CFTR inhibitors like inh172 have not been shown yet to reproducibly work in the ICM so that we adhered to the original protocol by Veeze et al. [29, 30] in this study.

In our study only six of the 20 subjects showed a stronger carbachol-evoked than histamine-evoked secretory response (Supplemental Table S2). The minute net residual chloride secretion in the ICM was DIDS-insensitive in most CF subjects and thus was ascribed to F508del CFTR activity and not to any alternative chloride and bicarbonate conductances. In other words, the variable presence of an alternative ion channel or transporter could not be the major confounder that caused the experimentally observed lack of association between the amount of band C F508del CFTR and chloride secretory activity in F508del homozygous rectal mucosa. The lack of association more likely reflect interindividual variations of the localization of band C F508del CFTR in the cell.

Detection of complex-glycosylated CFTR indicates that the protein passed the Golgi apparatus [38] but this does not imply that all mature CFTR is involved in ICM-detectable activity. Only a fraction of CFTR is located in the apical membrane where it secretes chloride into the extracellular space [10]. Wild type CFTR is endocytosed from and recycles back to the apical membrane, but F508del CFTR rapidly disappears from and does not return to the cell surface [10]. Most CFTR protein resides in the cell interior in a vesicle fraction beneath the intestinal brush border membrane [39], and in the Golgi apparatus and in the post-Golgi network [10, 40] including lysosomal compartments where CFTR has been reported to be involved in vesicular acidification [41]. This broad spatial distribution and the low half-life of the F508del mutant at the cell surface reasonably explain why the amounts of mature

glycoform and DIDS-insensitive chloride secretion are only weakly correlated with each other in the F508del condition.

In summary, our study revealed that the majority of investigated F508del homozygous subjects expressed low amounts of complex-glycosylated mature F508del CFTR and low residual F508del CFTR-mediated chloride secretory activity in the rectal mucosa. According to metabolic labelling F508del CFTR was synthesized and processed at wild type levels, but then rapidly degraded because the steady state concentration of complex-glycosylated F508del CFTR was only a few percent of that of CFTR in the intestine of healthy controls. The amount of mature F508del CFTR is probably even lower in tissues like the airways that do not regenerate as fast as the intestinal epithelium. Consistent with this interpretation, no mature F508del CFTR was detected in end-stage CF lungs, the major organ afflicted in CF.

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### **Appendix A:**

Supplementary data: Figures S1 and S2, Tables S1, S2 and S3

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### Figure Legends

Figure 1. Immunodetection of CFTR in lysates of non-CF rectal suction biopsies by immunoprecipitation (IP) and subsequent immunoblot.

A: After lysis of four fresh biopsies IPs were carried out with the pre-immune serum (PPI) R40 and R453 (lane b) or the polyclonal anti-CFTR Abs R40 and R453 (lanes a and c). CFTR was detected on the immunoblot of PAGE-separated IPs with the anti-CFTR mAbs 570 and 596 (lanes b and c) and the secondary Ab anti-mouse-IgG-HRP (lanes a-c). In lane c a strong complex-glycosylated C-band and a very weak mannose-rich B-band of CFTR was detectable, but not in lane a (secondary Ab) and in lane b (PPI and secondary Ab).

B: Treatment with Endo H (lanes b, d) deglycosylated the mannose-rich, but not the complex-glycosylated CFTR isoform in biopsies (lane a) and T84 cells (lane c). IPs were carried out with the polyclonal anti-CFTR Abs R40, R16/33 and R16/34. C: IPs of lysed biopsies of non-CF controls with different mixtures of polyclonal anti-CFTR Abs (lanes a, b: R40 and R453; lanes c, d: R40, R16/R33 and R16/R34) resulted in comparable normalized CFTR immunoreactive signals of band B and band C on blots. Each lane in panel C represents a separate immunoblot.

Figure 2. Immunodetection of CFTR in lung and distal intestine. Lysates of T84 cells (15.6 mg protein), non-CF rectal biopsies (0.64 mg), non-CF (6.6 mg) and F508del homozygous CF (6.4. mg) lung epithelium specimens were immunoprecipitated with polyclonal anti-CFTR Abs. CFTR was detected on the immunoblot of PAGE-separated IPs with the anti-CFTR CFTR mAbs 570 and 596. Please note the differential glycosylation patterns of band C CFTR in lung and rectal intestine and the about sevenfold lower normalized CFTR immunoreactive

signal in the lung sample than in the rectum sample. The F508del CFTR band B signal was there, but barely visible.

Figure 3. ICM tracings of rectal suction biopsies. An upward response denotes net chloride and bicarbonate secretion, a downward response denotes net potassium secretion unmasked in the absence of chloride secretion (observed in CF condition only) [21]. A chloride secretion in the presence of DIDS is attributed to CFTR. The ICM tracing of the non-CF subject (tracing A) shows the characteristic pattern of a healthy subject with large and nearly identical chloride secretory responses to carbachol and histamine of more than  $20 \mu\text{A}/\text{cm}^2$  together with a response to cAMP in the normal range. No net chloride secretory responses are seen in the tracing B of a CF subject who is homozygous for a CFTR null allele, the large out-of-frame deletion CFTRdele2,3(21kb).

The two tracings of F508del homozygous CF patients show either no net chloride secretion (tracing D) or residual chloride secretion that is attributable to CFTR because of the secretory response to DIDS/ histamine (tracing C). In tracing D after the response of carbachol a current drift was measured without any response to 8-Bromo-cAMP/Forskolin.

Figure 4. ICM tracing and CFTR immunoblot of the F508del homozygous CF subject 9.

A. The ICM tracing shows a high response to cAMP/forskolin being atypical for F508del homozygous CF. Although the CF-typical signature of a reverse response to carbachol was observed, the secretory response to cAMP was larger than the average response of a non-CF rectal mucosa. The CF-typical residual response to histamine indicates that F508del CFTR contributes to chloride secretion. B. The immunoblot shows a strong B-band of core-glycosylated and a weaker, but clearly visible C-band of complex-glycosylated F508del CFTR. The mature C glycoform is known to form the ion channel in the apical epithelial membrane.

Figure 5. Detection of CFTR or F508del CFTR in rectal mucosa of non-CF and F508del homozygous CF subjects. Four freshly excised rectal suction biopsies were examined in their responses to secretagogues by ICM and immediately thereafter for their CFTR immunoreactive signals by immunoblot of IPs following a standardized protocol for sample processing and analysis. Each lane represents a separate immunoblot. In 4 of 5 F508del homozygous CF samples the mannose-rich (lanes b, c, d and f) and complex-glycosylated F508del CFTR isoforms (lanes c-f) were clearly detected by mAbs 570 and 596. The amount of DIDS-insensitive residual chloride secretory activity did not correlate with signal intensity of the C-band (see Figure 6, Supplemental Figure S2 and Supplemental Table S2 for ICM and CFTR protein data on the complete cohort).

Figure 6. Contents of CFTR B-band and C-band in immunoblots of SDS PAGE separated anti-CFTR IPs of rectal mucosal biopsies of F508del homozygous subjects (con, non-CF control). The normalized density indicates the immunoreactive CFTR signal on a X-ray film that had been exposed for one minute to the immunoblot covered with ECL Advance. B- and C- F508del CFTR glycoforms were detected in highly variable amounts and C/B ratios (see Table 1).

Figure 7. Autoradiographs of metabolically labelled rectal biopsies from non-CF and F508del (F508d) homozygous CF subjects.

A. Analysis of newly synthesized CFTR. After ICM and metabolic labelling for 5 h (lanes a, c [22]), 16 h (lane b, [22]) and 15 h (lanes d-g) the biopsies were lysed and immunoprecipitated with a mixture of anti-CFTR Abs (see headings above the individual lanes). The IPs were separated by 5% SDS-PAGE and the dried gels exposed to Biomax MR1 films. The complex-glycosylated C-band of CFTR or of F508del CFTR was visible in lanes a, b, c and g as a

broad band and in lanes d, e and f as a double band. The B-band was detected in all lanes. In lane c, d, e and lane f an additionally CFTR immunoreactive diffuse band marked as B\*-band was visible, but not in lane a, b and g. Lanes a, b, c were reproduced from Figure 2 of our previous publication [22] in order to demonstrate that immunoprecipitations with different anti-CFTR antibodies yielded similar patterns of metabolically labelled CFTR bands in the autoradiogram.

B. Comparison of the biosynthesis of CFTR and sucrase-isomaltase (SI) in rectal biopsies from a F508del homozygous individual with CF (lanes c, d) and a non-CF healthy control (lanes a, b). After ICM and metabolic labelling, the biopsies were lysed. The lysates were sequentially immunoprecipitated with mixtures of anti-CFTR Abs and then of anti-SI Abs (see headings above the individual lanes). SI was chosen as the reference marker protein because the protocol of metabolic labelling of minute intestinal biopsies and subsequent IPs had been initially set up for the analysis of SI [24, 25].

Figure 8 A. Endo H digestion of metabolically labelled non-CF rectal biopsies. Treatment with Endo H deglycosylated the mannose-rich bands B and B\*, but not the complex-glycosylated band C (lane b) in biopsies.

B. F508del CFTR analysis by metabolic labelling or immunoblot of biopsies from the same subject. Metabolic labelling of two rectal biopsies yielded faint C- and B-bands of F508del CFTR (lane a), whereas the immunoblot of four biopsies taken six years later revealed a strong B-band, but no C-band (lane b).

**Table 1. Ratio of the complex-glycosylated CFTR C-band to the mannose-rich B-band in immunoblots of lysed rectal biopsies from F508del homozygous CF subjects and a non-CF subject.**

Subject no.	Density of B-band [od/mm <sup>2</sup> ]	Density of C-band [od/mm <sup>2</sup> ]	C/B ratio
control	1011711	6953689	7
8	1581099	0	0
9	769415	389148	0.5
10	494162	975820	2
11	313578	182694	0.6
12	236680	185384	0.8
13	151372	695356	5
14	62335	629557	10
15	3354	7336	2
16	1253740	0	0
17	598796	0	0
18	23482	931195	40
19	851444	343543	0.4
20	4131	8562	2

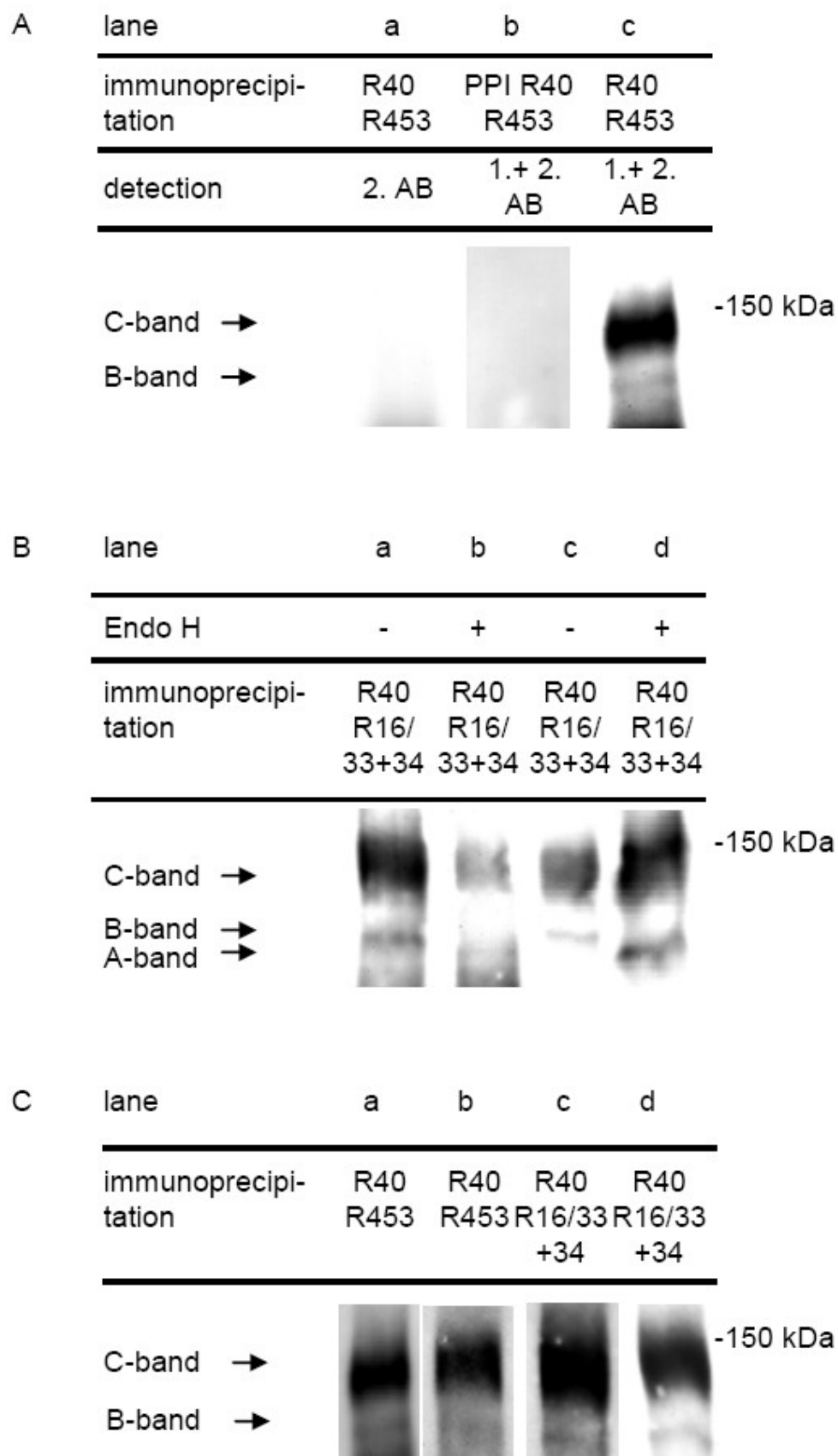


Fig. 1

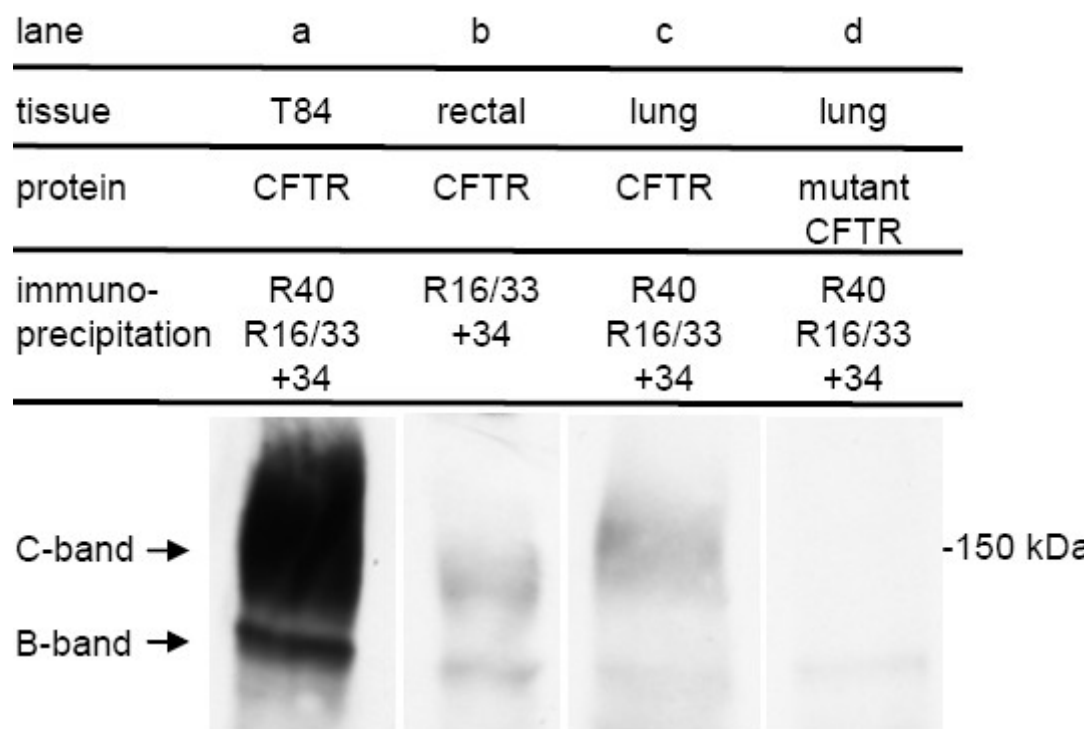


Fig. 2

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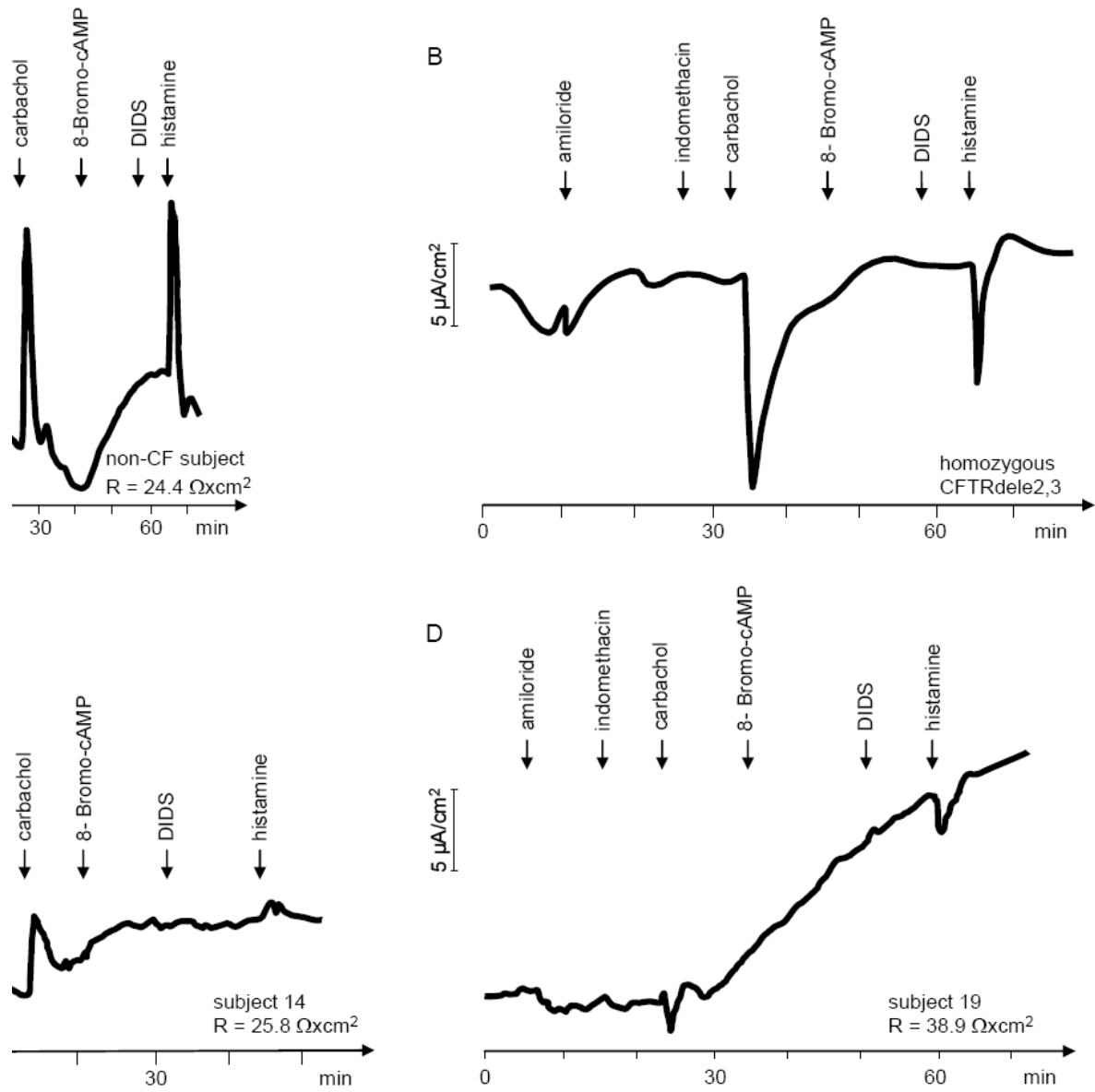


Fig. 3

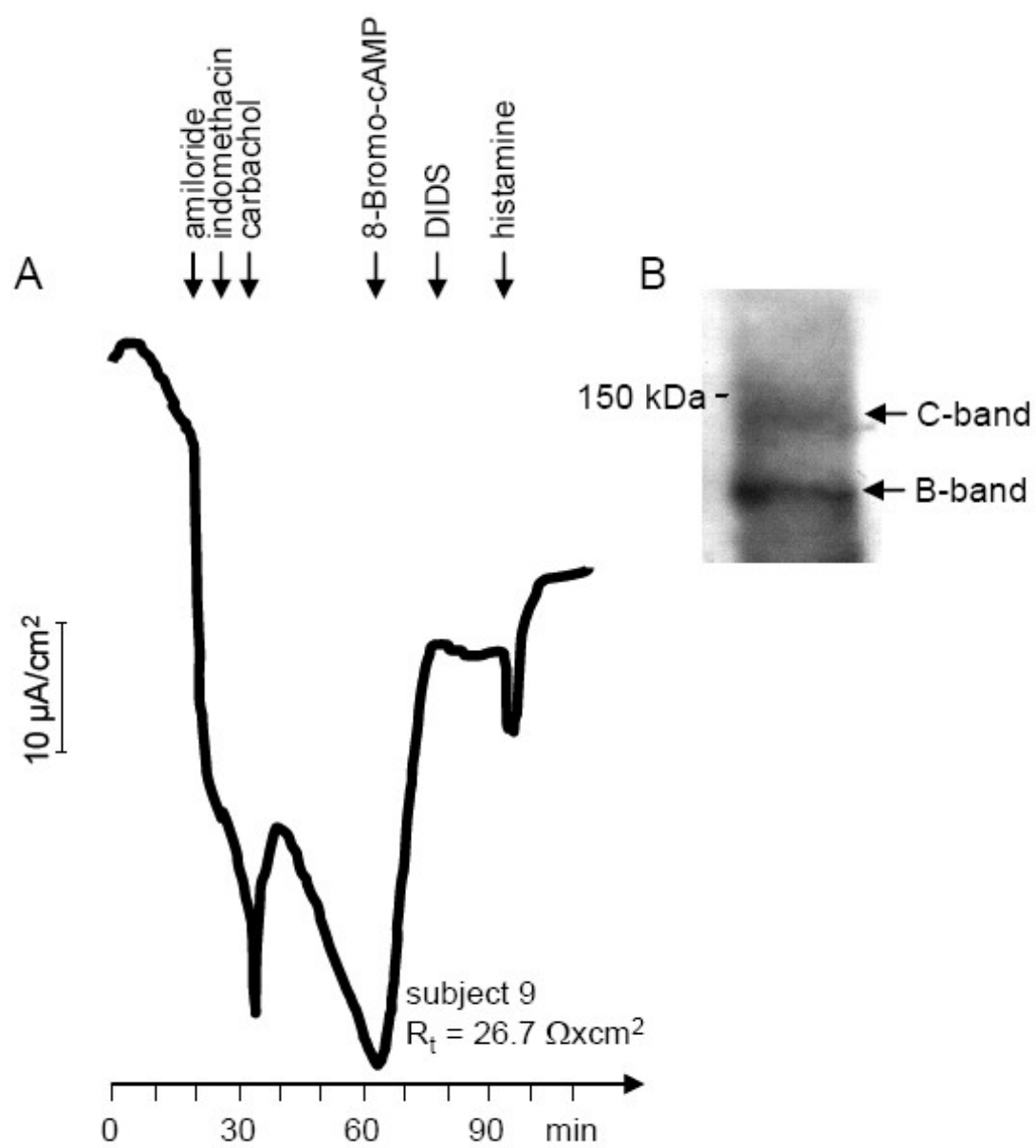


Fig. 4

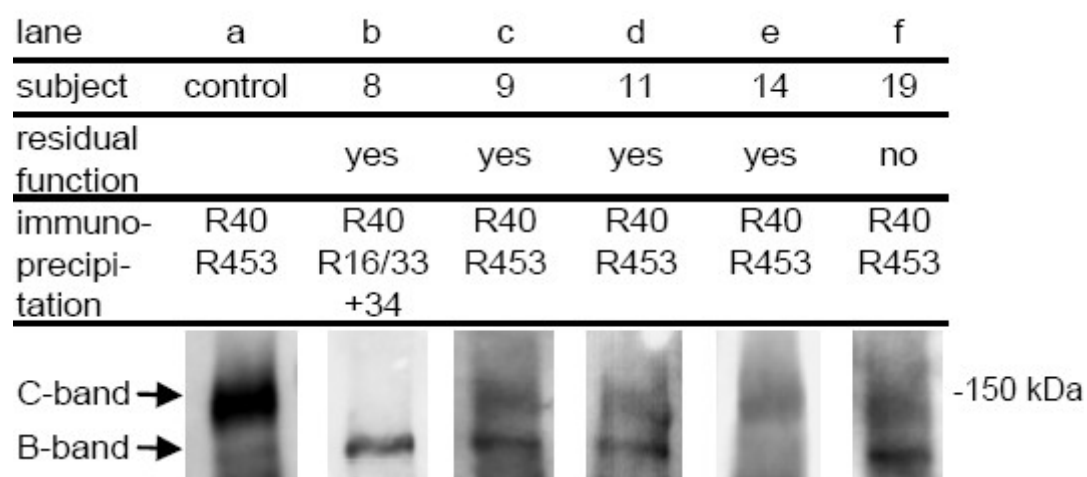


Fig. 5

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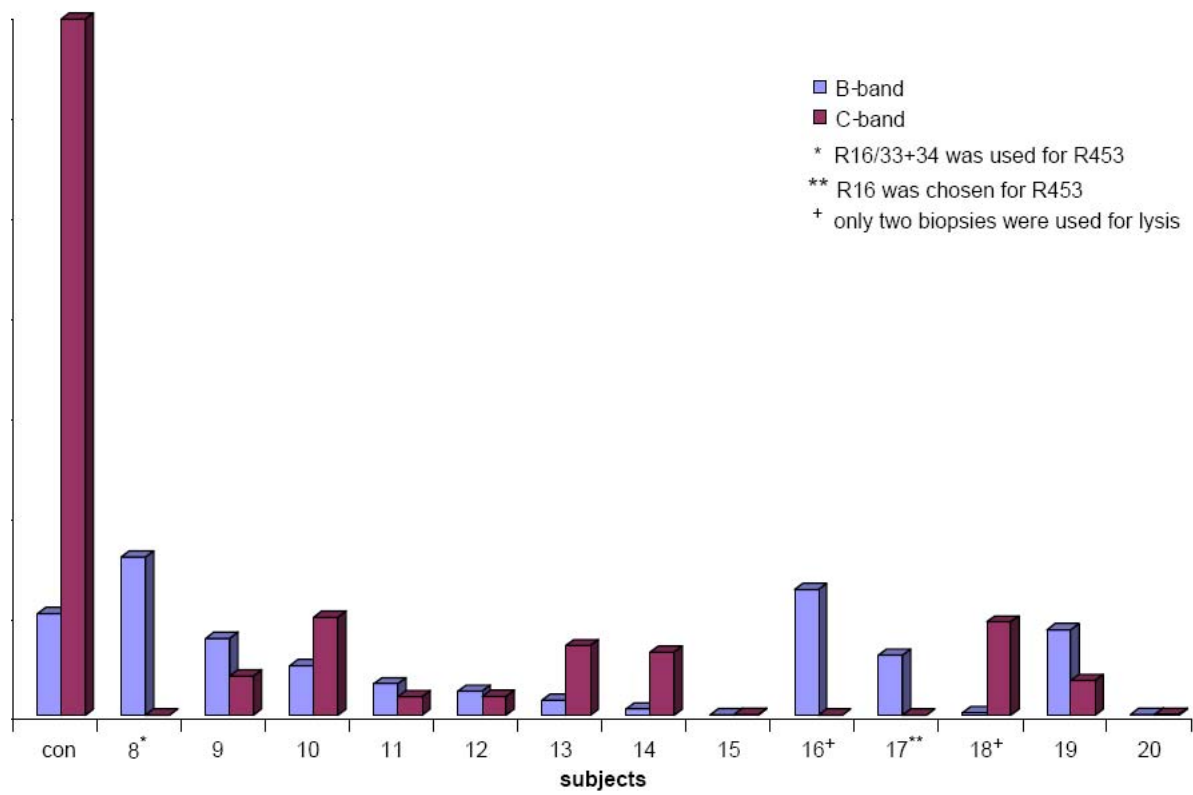


Fig. 6.

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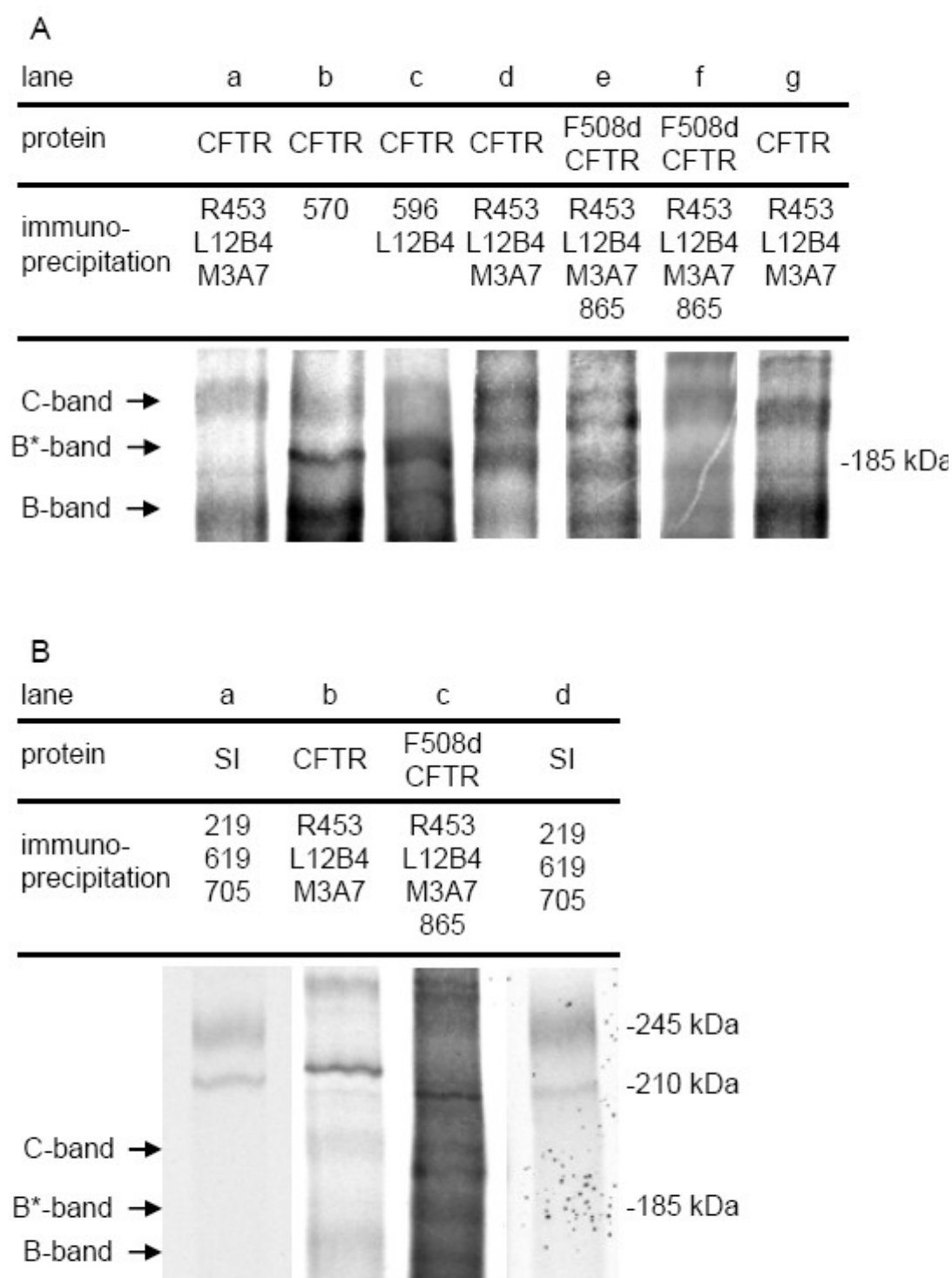


Fig. 7

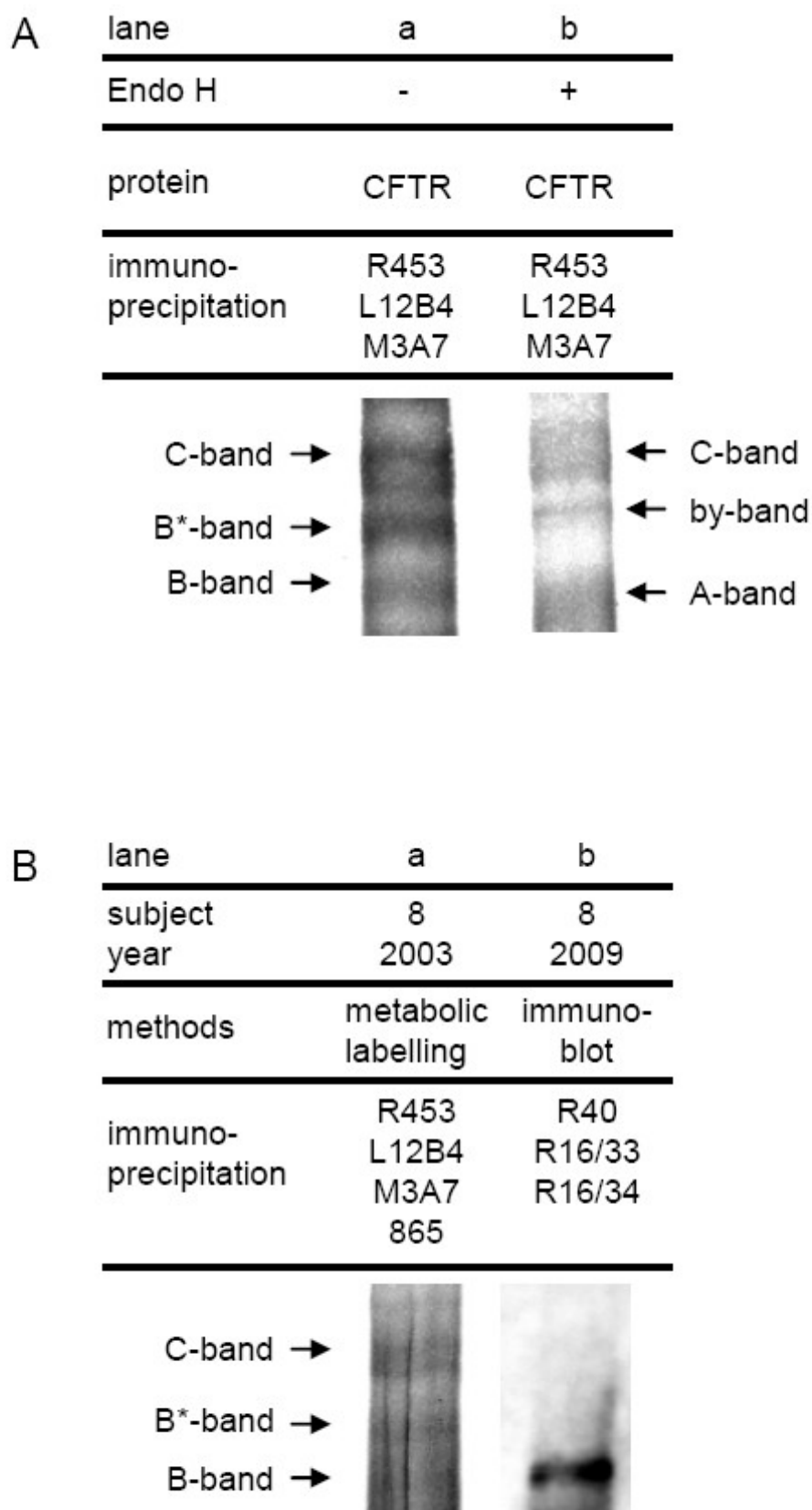


Fig. 8