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Rapid report

Ex vivo biochemical analysis of CFTR in human rectal biopsies

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Abstract

This report describes the first biosynthetic analysis of the cystic fibrosis transmembrane conductance regulator (CFTR) in freshly excised human rectal biopsies. Expression of functional CFTR was assessed by intestinal current measurement (ICM) prior to biosynthetic studies. Several structural features of CFTR are found to be comparable to those established in CFTR-expressing cell lines. Interestingly, maturation of CFTR increases substantially in tissue incubated at 26 °C. Our data provide a solid basis for future studies on the characterisation of CFTR in pathological cases.

Keywords: CFTR; Metabolic labelling; Immunoblot; Human tissue

Cystic fibrosis (CF) is the most common severe disease of autosomal recessive inheritance in the Caucasian population. It is caused by mutations in the CF transmembrane regulator (*CFTR*) gene, which encodes a cAMP-regulated Cl^- channel that is expressed in the apical plasma membrane of exocrine epithelia [1,2]. Most of our knowledge about human CFTR resides on CFTR-expressing model systems such as immortalized or recombinant cell lines. Much less is known about the structure, biosynthesis, processing and trafficking of CFTR in native human tissue. CFTR biochemistry and function in human tissue have been addressed in a few studies by immunocytochemistry [2–9], immunoblot [6–9] and ion transport analysis [10–13]. The limited amount of CFTR expressed in native tissues as well as the lack of sufficiently sensitive and specific anti-CFTR antibodies [6] have apparently hampered metabolic labelling analysis in native human tissue.

Here, we report on an ex vivo metabolic labelling [14] and immunoblot study on freshly excised human rectal biopsies. In the present study 2 to 4 specimens per volunteer were freshly excised according to safety and ethical precautions. This study was approved by the ethical committee at the Medical School Hannover, Germany and volunteers gave their written informed consent. The suction device yields <2 mm in diameter epithelial specimens that are devoid of submucosa and/or muscle [11].

Rectal biopsies are epithelial in nature, express CFTR as a major chloride channel and are accessible by a harmless and non-invasive procedure [6,12]. Moreover, a standardized intestinal current measurement (ICM) protocol enables to determine tissue viability and CFTR activity of the individual freshly excised biopsy [11,12,16,17]. CFTR is recognized in ICM by its electrophysiological profile of DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid)-insensitive secretory responses to forskolin/cAMP and cholinergic agonists (i.e., carbachol and histamine, Fig. 1A) [12,17]. Fig. 1 shows the tracings of tissues obtained from a volunteer (Fig. 1A) and for comparison from a CF patient lacking any chloride secretion (Fig. 1B) [18]. Please note that the signal evoked by addition of carbachol, 8-bromo-cAMP/forskolin or histamine represents a superposition of chloride and potassium efflux. An upward response indicates the net chloride efflux (Fig. 1A), whereas the downward response reflects the potassium efflux in individuals lacking CFTR (Fig. 1B) [11].

Prior to metabolic labelling or immunoblot analysis, the biopsies from all 22 volunteers between 2 and 60 years were

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examined by the ICM protocol described in detail by Bronsveld et al. [12]. Normal chloride secretory responses upon exposure to carbachol, forskolin or histamine insensitive to DIDS were detected indicating normal activities of CFTR (Table 1, Fig. 1A). All biopsies that successfully passed the ICM experiment, were included into the biochemical analysis.

During the 2 h of ICM the biopsy samples were incubated in Meyler buffer that is supplemented with glucose as the only carbon source. Hence, immediate biosynthetic labelling of two rectal biopsy specimens with ^{35}S-cysteine and ^{35}S-methionine could be carried out without further preincubation in cysteine and methionine-free medium and was followed by detergent extraction of the membrane proteins. Immunoprecipitation of the extracts with the polyclonal anti-CFTR antibody R453 and the mononoclonal anti-CFTR antibodies M3A7 and L12B4 [2,15] revealed two main molecular forms of CFTR upon analysis by SDS-PAGE. These forms had approximate apparent molecular masses of ~165 kDa and 195 kDa (Fig. 2, lane a) and correspond therefore to the mannose-rich (B-band) and complex-glycosylated (C-band) isoforms of CFTR. The identity of the biosynthetic glycoforms in the biopsy samples was assessed by direct comparison with the CFTR isoforms immunoprecipitated with R453 antibody from T84 cells and treated with endo-β-*N*-acetylglycosaminidase H and endo-β-*N*-acetylglycosaminidase/*N*-glycosidase F (data not shown). As a suitable control for efficiency of the metabolic labelling, proper processing of glycoproteins and hitherto integrity of the tissue during labelling we utilized the membrane glycoprotein sucrase-isomaltase (SI) that has been extensively characterized in human duodenal biopsy specimens [19]. In intestinal biopsy specimens, two major biosynthetic forms of SI are revealed, a mannose-rich 210 kDa (Pro-SI$_h$) precursor and a 245-kDa

Table 1
ICM data of the rectal biopsy specimens obtained from 22 volunteers (2 to 60 years of age) examined in this study for CFTR protein

	Mean±standard deviation	Reference values
Basal transepithelial resistance	23.9±9.3 Ω · cm^2	
Basal I_{sc}	55.8±30.1 μA/cm^2	
ΔI_{sc} amiloride	−5.2±8.5 μA/cm^2	
ΔI_{sc} carbachol	29.9±13.5 μA/cm^2	43.3±17.6[a] μA/cm^2 [12] 35.1±2.6[b] μA/cm^2 <18 years old [16] 22.4±4.3[b] μA/cm^2 >18 years old [16]
ΔI_{sc} 8-bromo-cAMP/forskolin	17.4±14.6 μA/cm^2	
ΔI_{sc} histamine	29.8±22.4 μA/cm^2	39.3±19.3[a] μA/cm^2 [12]

[a] Mean±standard deviation.
[b] Mean±standard error of mean.

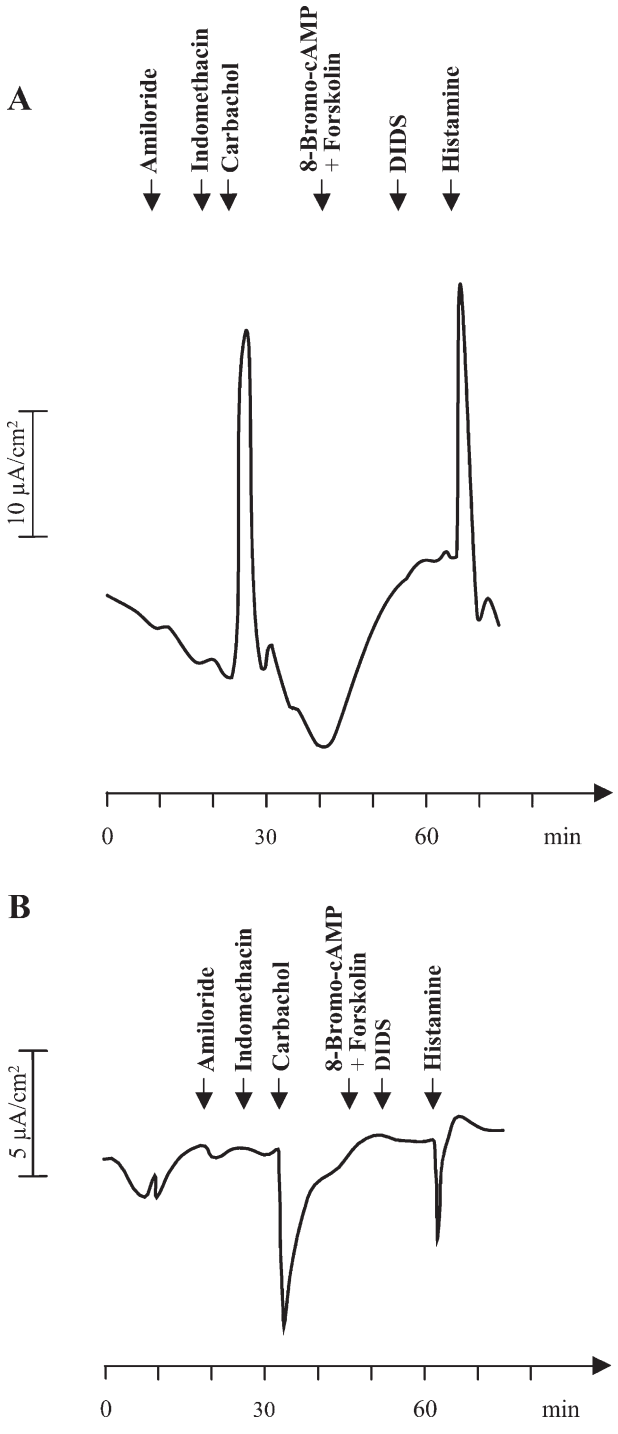

Fig. 1. ICM tracings of rectal suction biopsies obtained from (A) a healthy volunteer and (B) a CF-patient. An upward response denotes net chloride secretion (observed seen in panel A, but not in panel B), a downward response denotes net potassium secretion (observed in the CF condition only) [11]. ICM [12]: The electrogenic transport of ions across the intestinal epithelium was measured as short circuit current (I_{sc}) on biopsies of the rectal mucosa and mounted into an adapted micro Ussing chamber with an aperture of 1.13 mm^2. The tissue was perfused in Meyler Buffer (126.2 mmol/l Na^+, 114.3 mmol/l Cl^-, 20.2 mmol/l HCO_3^-, 0.3 mmol/l HPO_4^{2-}, 0.4 mmol/l $H_2PO_4^-$, 10 mmol/l HEPES, 10 mmol/l glucose, pH 7.4) at 37 °C and gassed with 95% O_2 and 5% CO_2. Basal transepithelial resistance of the tissue was determined by measuring voltage response to pulse currents of 1 μA, and basal I_{sc} was calculated from the basal transepithelial resistance and the open-circuit transepithelial potential difference. The tissue was subsequently short-circuited by voltage clamps, resulting in a transepithelial potential difference of zero. After equilibration, specific modulators were added to the mucosal (***M***) and/or serosal (***S***) sides of the biopsy: 1. amiloride (1×10^{-4} M, ***M***), 2. indomethacin (1×10^{-5} M, ***M+S***), 3. carbachol (1×10^{-4} M, ***S***), 4. 8-bromo-cAMP (1×10^{-3} M, ***M+S***) and forskolin (1×10^{-5} M, ***S***), 5. DIDS (2×10^{-4} M, ***M***) and 6. histamine (5×10^{-4} M, ***S***). All chemicals were obtained from SIGMA-Aldrich, Steinheim, Germany.

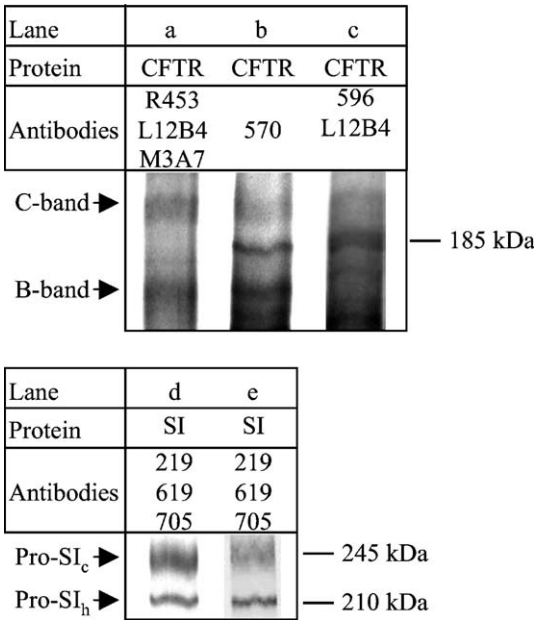

Fig. 2. Metabolic labelling of rectal biopsies. After ICM two rectal suction biopsy specimens were placed on a stainless steel grid and labelled biosynthetically with 15.4 MBq [^{35}S]cysteine and 18.5 MBq [^{35}S]methionine (Amersham Biosciences) in methionine and cysteine deficient D-MEM medium (GIBCO) supplemented with streptomycin and penicillin (GIBCO) following our protocol established for the analysis of lactate-phlorizin hydrolase [24]. The biopsies were incubated at 37 °C for 5 (lanes a, c, d, e) or 16 h (lane b). The biopsy specimens were homogenized in the presence of 10 mM iodoacetamide, 20 mM PMSF, 20 μg/ml pepstatin and antipain, 100 μg/ml leupeptin and aprotonin and 500 μg/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 × g, 4 °C, 20 min) the supernatant was incubated with protein A-sepharose (PAS, Amersham Biosciences) for 60 min. The immunoprecipitation was carried out overnight except with the monoclonal antibodies M3A7 (1:80, Chemicon, Temecula, USA), L12B4 (1:80, Chemicon, Temecula, USA) and the polyclonal antibody R453[1] (1:60) in the presence of PAS and protein G-agarose (Sigma-Aldrich, lane a) or with the monoclonal antibody 570 linked to protein A agarose for (lane b, immunoprecipitation for 4 h), with the monoclonal antibodies 596 (1:500) and L12B4(1:80) with protein G-agarose and PAS (lane c). The monoclonal antibodies 570 and 596 were generously provided by J. R. Riordan. SI in lane d and e was immunoprecipitated for 2 h with a mix of monoclonal antibodies HBB1/219, HBB 2/619 and HBB 3/705, generously provided by H. P. Hauri (Basel, Switzerland) and E. E. Sterchi (Berne, Switzerland), in the presence of PAS. All pellets were washed several times with washing buffers [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]. Immunoprecipitates were separated by 5% SDS-PAGE. The gel was vacuum-dried and exposed to Biomax MR1 film (Kodak).

complex glycosylated mature (Pro-SI$_c$) form (Fig. 2, lanes d and e). Ideally 5% acrylamide gels are routinely used to ensure a high resolution of the high-molecular weight isoforms of SI [19]. Molecular weights of CFTR isoforms were calculated by comparison with the molecular weights of β-galactosidase, α_2-macroglobulin, myosin and SI.

[1] The polyclonal antibody R453 was raised against two synthetic peptides corresponding to amino acid 1–14 (MQRSPLEKASVVSK) of the N-terminus of CFTR and to amino acid 1466–1480 (ALKEETEEEVQDTRL) of the C-terminus of CFTR using the double X-system (Eurogentec, Seraing, Belgium). After the third booster the serum was purified by affinity chromatography using protein A-Sepharose.

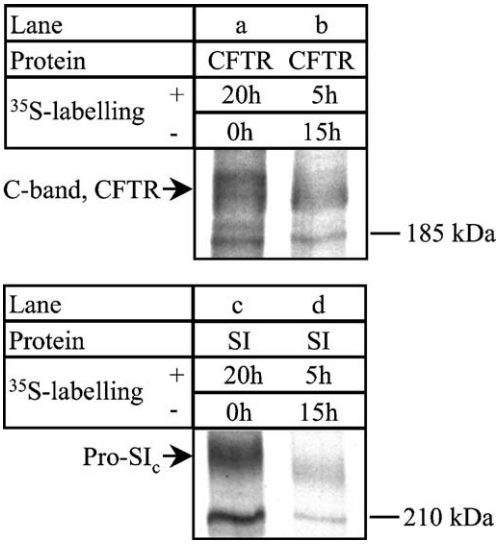

Fig. 3. Stability of metabolically labelled CFTR and SI. Rectal biopsies were metabolically labelled with ^{35}S-cysteine and ^{35}S-methionine in methionine and cysteine deficient D-MEM medium either continuously for 20 h (lanes a, c) or for 5 h followed by incubation for 15 h in complete, non-radioactive medium D-MEM/F-12 (GIBCO) supplemented with 5% fetal bovine serum (GIBCO, lanes b, d). Lysis of tissue was performed with 0.10% SDS, followed by immunoprecipitation with R453, L12B4 and M3A7. Separation by 5% SDS-PAGE and detection by autoradiography. Metabolic labelling, lysis, immunoprecipitation, separation and detection are described in detail in Fig. 2.

To confirm the identity of the CFTR bands immunoprecipitated by an antibody mixture of R453, M3A7 and L12B4, alternatively the monoclonal anti-CFTR antibody 570 linked to protein A-agarose or a mixture of the monoclonal anti-CFTR antibodies 596 and L12B4 were chosen. Both immunoprecipitations (Fig. 2, lanes b and c) showed a band of the expected 195 kDa in size. The pattern of further CFTR immunoreactive bands of lower molecular weight, however, differed with the antibody mixture used indicating that the epitopes are not equally accessible in each CFTR isoform. Most efficient immunoprecipitations were obtained by utilizing a mixture of R453 with

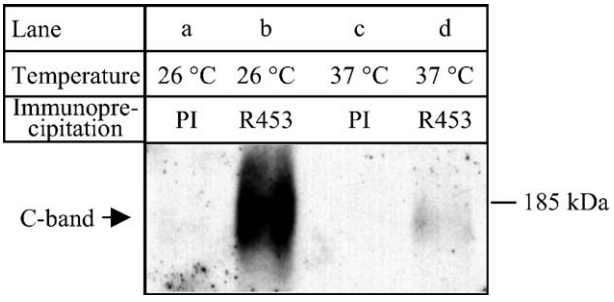

Fig. 4. Stability of CFTR in human rectal tissue at 26 and 37 °C. After ICM two biopsies from the same proband were incubated overnight at either 26 or 37 °C in complete D-MEM/F-12 (5% fetal bovine serum). The lysed biopsies were incubated with the pre-immune serum (PI) from R453 for 2 h. Immunoprecipitaton followed with R453 (1:60) in the presence of PAS. The immunodetection was carried out in the presence of the monoclonal antibodies 596 and 570 (1:1000) and anti-mouse IgG HRP (Amersham Bioscience) using ECL Advance (Amersham Bioscience) as detection reagent. Lysis and separation are described in detail in Fig. 2.

Table 2
Survey of the experiments carried out with ^{35}S-labelled human rectal biopsies

Type of experiment	No. of subjects	No. of biopsies	Total no. of biopsies	Presence of C-band CFTR	Figure [a]
Optimization of detection of ^{35}S-labelled CFTR	3	3×2	6	2 of 3 subjects	
^{35}S-labelled CFTR	8	8×2	16	8 of 8 subjects	Fig. 2, lane a
Half-life of ^{35}S-labelled CFTR	3	3×4	12	5 of 6 experiments [b]	Fig. 3
Incubation at 26°C and 37°C in parallel	3	3×2	6	2 of 3 subjects	Fig. 4
Test of anti-CFTR Ab596	4	4×2	8	4 of 4 subjects	Fig. 2, lane c
Test of anti-CFTR Ab570	1	1×2	2	1 of 1 subject	Fig. 2, lane b
Total	22	50	50		

[a] A representative example of this type of experiment is shown in the respective figure.
[b] For each volunteer, two biopsies were labelled with ^{35}S continuously for either 5 h or 20 h, respectively, and then processed, whereas the other two biopsies were labelled with ^{35}S for 5 h followed by incubation for 15 h with non-radioactive medium.

the monoclonal antibodies L12B4 and M3A7 (Fig. 2, lane a). The latter two monoclonal antibodies have been used previously for immunoprecipitation of CFTR from cell lines [20,21], but were not efficient in immunoisolating CFTR from human tissue (van Barneveld A., unpublished data).

To gain information about the maturation and turnover of the CFTR protein bands in human rectal tissue, we labelled two biopsies for 5 h followed by incubation with non-radioactive medium for 15 h. As shown in Fig. 3, a qualitative difference in the composition of the C-band of CFTR becomes evident during the prolonged incubation time (lane b). While the C-form appears as a heterogeneous doublet by continuous labelling (lane a), the intensity of the upper band of the doublet had decreased substantially 15 h after labelling had been terminated and the C-form is converted to one single slightly diffuse band of a similar size as the lower band of the doublet (lane b). A qualitative difference in the biosynthetic features of the control glycoprotein SI occurs as well. Here, the complex glycosylated form of SI in the 5 h labelling time point was also processed to an isoform of a reduced apparent molecular weight (compare lane c with d). These data clearly show that the final maturation events in the biosynthetic life cycle of the CFTR and SI proteins implicate processing, most likely truncation, of the complex glycosylated forms of these proteins. At least for CFTR, this processing ends up with a more homogeneous glycosylation pattern.

In cell lines, it was shown that the protein levels of CFTR are higher at 26 °C than at 37 °C in mutant F508del CFTR, which to some extent has been also shown for wild type CFTR [22]. We examined this aspect by immunoblotting of CFTR with R453 using detergent extracts of biopsy samples that have been incubated overnight at 26 °C or 37 °C (Fig. 4). Indeed, incubation at 26 °C resulted in a dramatic increase in the protein levels of CFTR as assessed by the stronger signal of complex-glycosylated CFTR (lane b) as compared to the protein isolated from the tissue incubated at 37 °C (lane d). This observation is not due to differential viability of the tissue at the two temperatures because under our ex vivo culture condition intestinal biopsies are known to be metabolically active for at least 24 h [19,23,24]. Hence, the differential steady state concentrations of the mutant F508del CFTR at 26 °C and 37 °C [20] are also applicable to wild type CFTR. The immunoblot analysis with the monoclonal CFTR antibodies 570 and 596 confirmed the size of complex-glycosylated band C of CFTR determined in metabolic labelled rectal biopsies (Fig. 4). Table 2 provides information about the number of biopsies and subjects applied to each type of experiment. Further, CFTR was identified in 19 out of 22 analyzed specimens which is not significantly different from a 100% detection rate ($P = 0.12$).

The aim of this study was to establish reproducible and sensitive methods for the ex vivo analysis of human CFTR by metabolic labelling or immunoblot whereby the CFTR expressing tissue should be easily accessible by a safe, painless and non-invasive procedure. These criteria apply to rectal suction biopsies or nasal brushings to harvest epithelial cells. However, the cell population from nasal brushings is heterogeneous and hence this source is only suited for functional or immunocytochemical single cell analysis [13,25,26], but not for CFTR biochemistry.

Ethical considerations limited the maximal number of rectal biopsies to be retrieved from one proband to four. Accordingly, our protocols had to be optimized in their sensitivity and specificity of immunochemical detection of CFTR. To this end, two biopsies turned out to be sufficient to visualize CFTR by immunoblot or metabolic labelling. The key to improving the sensitivity and specificity was the combination of two or more high-affinity CFTR antibodies. Mall and colleagues [8,9] were the first to demonstrate that strong immunoreactive CFTR signals with a high signal-to-noise ratio can be achieved if CFTR is precipitated with a polyclonal antibody and the blots are probed with a cocktail of monoclonal antibodies which recognize separate CFTR epitopes. In our hands, the combination of our polyclonal antibody R453 for immunoprecipitation with the highly sensitive monoclonal antibodies 570 and 596 developed for immunoblot in J. R. Riordan's laboratory visualized immunoreactive CFTR bands from two biopsies (Fig. 4).

In summary, we report for the first time on metabolic labelling of CFTR ex vivo in human tissue. Two rectal biopsies were sufficient to detect CFTR. The data indicate that the ex vivo analysis of CFTR processing and trafficking should be possible. A comparative study on biopsies retrieved from F508del homozygous CF patients and non-CF healthy subjects would be first priority.

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