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A comparison of 14 antibodies for the biochemical detection of the cystic fibrosis transmembrane conductance regulator protein

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Abstract

Interest in the biochemical detection of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein followed soon after cloning of the gene and prediction of the protein structure. Ever since, antibodies (Abs) have been produced and used to detect CFTR in both heterologously and endogenously expressing cells and tissues. Although designed to be sensitive and specific, these Abs produce, in most cases, unsatisfactory results when used for the biochemical detection of CFTR either by Western blot or by immunoprecipitation. The lack of Abs that can reliably detect the CFTR protein is a major constraint to studies of CF. We compared 14 different Abs for their ability to detect CFTR in both stably transfected and endogenously expressing cell lines.

Keywords: CFTR; Antibody; Western blot; Immunoprecipitation

1. Introduction

The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein is an integral membrane glycoprotein that is mainly expressed at the apical membrane of epithelial cells where it functions as a cAMP-stimulated chloride (Cl^-) channel [1]. CFTR is a member of the ATP-binding cassette (ABC) transporter superfamily, has 1480 amino acid residues and comprises several domains: two transmembrane domains (TM1 and TM2) with six membrane-spanning segments each, two nucleotide binding domains (NBD1 and NBD2) and a regulatory (R) domain (see Fig. 1) [1]. The R domain has been shown to act as a regulator of channel gating through the phosphorylation of its serine residues, a property that has also been used for the detection of CFTR protein by [^{32}P] phosphorylation assays [2]. As is common for glycoproteins, CFTR is co-translationally integrated into the endoplasmic reticulum (ER) membrane where it is core-glycosylated by N-linking of glycans to asparagine residues N894 and N900 [2]. Following ER exit along the secretory pathway through the Golgi apparatus, the CFTR polypeptide chain undergoes further post-translational modifications to produce the fully glycosylated or mature form [3]. According to its glycosylation status, three different forms of the protein can be distinguished by SDS-PAGE analysis, namely, (1) the newly synthesized non-glycosylated primary polypeptide, usually known as band A of 130 kDa (only biochemically detected upon treatment with glycosidases), (2) the ER core-glycosylated, or immature form of the protein, also known as band B, of 150 kDa, and (3) the fully-glycosylated, or mature form of CFTR, also known as band C, with an apparent molecular mass of 170–180 kDa [3]. Monitoring the glycosylation status of CFTR is thus a convenient way to assess its intracellular processing and to distinguish

Abbreviations: Aa, aminoacid; Ab, antibody; ABC, ATP-binding cassette; AT, alternative initiation (site); BHK, baby hamster kidney (cells); CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; CHO, chinese hamster ovary cells; IP, immunoprecipitation; mAb, monoclonal antibody; NBD, nucleotide binding domain; pAb, polyclonal antibody; 4-PBA, sodium 4-phenylbutyrate; PBS, phosphate buffered saline; PKA, protein kinase A; RT, room temperature; sAb, secondary antibody; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TM, transmembrane domain; WB, western blot.

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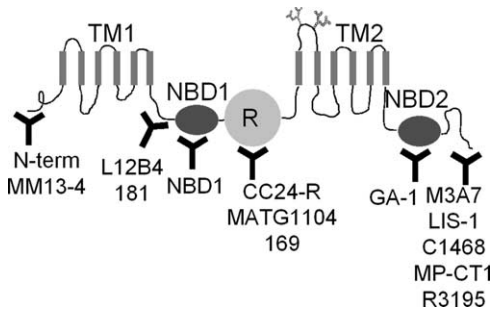

Fig. 1. Schematic diagram of CFTR protein depicting the different domains and indicating the epitopes against which the various Abs used in this study were raised.

the wild-type (wt) protein from processing mutants like F508del, the most common mutation.

Several antibodies (Abs) have been produced and used to biochemically detect CFTR from different sources, including heterologous expression systems, cell lines constitutively synthesizing the protein and also native tissues. Due to the very low levels of expression, biochemical detection of CFTR in the latter remains difficult unless extracts of membrane proteins are produced first [4–7]. Despite this low abundance, CFTR can be successfully detected in native tissues by immunocytochemistry. We have previously reported the applicability of different antibodies for the immunocytochemical detection of CFTR both in native tissues and freshly isolated native cells [8–10].

A variety of factors influence the biochemical detection of CFTR. These may depend on the characteristics of the sample, such as CFTR expression levels in a given cell line, the copy number of CFTR genes in transfected cell lines, or the cell polarity. Other factors influencing detection relate to the characteristics of the Abs themselves, namely the IgG concentration in the antiserum, whether they were affinity purified or their intrinsic specificity and sensitivity. Here, we address issues of sample variability by testing a panel of nine cell lines (one of these in both polarized and non-polarized states), including some with endogenous CFTR expression and others with transgene expression. We studied issues related to the sensitivity and specificity of anti-CFTR Abs for CFTR detection by Western blot (WB) and immunoprecipitation (IP) by comparing 14 different anti-CFTR Abs, both poly and monoclonal, from commercial sources and produced by research labs.

2. Materials and methods

2.1. Cells and culture conditions

A total of nine different cell lines were used, corresponding mainly to three categories: (i) cell lines not expressing CFTR (negative controls), (ii) negative lines stably transfected with either wt- or F508del-CFTR cDNAs, and (iii) cell lines endogenously expressing either wt- or F508del-CFTR (i.e. derived from tissues of non-CF or CF individuals, respectively). The first group included chinese hamster ovary (CHO) K1 cells (CCL61) and baby hamster kidney (BHK) cells, both from ATCC (Manassas, VA, USA). The second group included the cell lines from the first group, but stably transfected with wt- or F508del-CFTR, produced and cultured as previously described [11,12]. CHO cells express less CFTR than BHK cells. The third group of cells included the human bronchial cell line 16HBE14o-, isolated from a non-CF individual [13] and the human submucosal gland cell line Calu-3, isolated from a non-CF individual [14]. 16HBE14o-cells were grown under two different conditions, either directly on regular (plastic) culture flasks or on flasks pre-coated with a collagen film that induces cellular differentiation and polarization. Polarized cells have been reported to increase CFTR expression levels [15–17]. Although not shown, analyses were also performed in the following cell lines: the human bronchial epithelial cell line IB3-1 (*CFTR* genotype F508del/W1282X) [18], the human colonic cell line HT29, endogenously expressing wt-CFTR [19], and the human tracheal cell line ΣCFTE29o- (*CFTR* genotype F508del/F508del) [20]. Sodium phenylbutyrate (4-PBA), which has been reported to increase transcription in general and CFTR expression in particular [21–23] by acting as an inhibitor of histone deacetylase, was used for 24 h at 2 mM to increase CFTR expression.

2.2. Generation of a polyclonal anti-CFTR antibody

A peptide corresponding to CFTR amino acid (aa) residues 1468–1480 (KEETEEEVQDTRL) was synthesized, HPLC-purified, conjugated to thyroglobulin and used to inject two rabbits (*Research Genetics*, Huntsville, AL, USA). Affinity purification of sera from the second bleed or the terminal bleed (Lis-1) was performed using a column of the peptide conjugated to epoxy-activated sepharose beads (*Sigma Genosys*, Cambridge, UK). The affinity columns were first washed with 50 mM NaCl, 50 mM Tris–HCl pH 8.0 and then incubated for 30 min with antiserum diluted two-fold with PBS. The column was then washed once with the same buffer before eluting the Ab with 0.1 M glycine pH 2.5 in 0.5 ml fractions. Each fraction was neutralized with Tris. The Ab-rich fractions were subsequently desalted and concentrated in Centricon YM-10 columns with a 10,000 MW cut-off (*Millipore*, Bedford, MA, USA).

2.3. Antibodies

Besides Lis-1 pAb (above), the following rabbit polyclonal anti-CFTR antibodies (pAbs) were tested (see Fig. 1 and Tables 1 and 2): Ab169, generated against aa residues 724–746 [24], Ab181, generated against aa residues 386–412 [24] AbN-term-Birmingham, generated against

Table 1
Applicability of different antibodies for the biochemical detection of CFTR by Western blot

Ab	Type[a]	Epitope	Dilution	Applicability[b]							Origin	Ref.
				CFTR transfected[c]		Endogenously expressing CFTR						
				CHO	BHK	Calu-3	16HBE	HT29	IB3-1	ΣCFTE		
M3A7	MC	C-term (1197–1480)	1:1000	+++	+++	+++	+	ND	−	ND	Chemicon	[29]
L12B4	MC	Pre-NBD1 (386–412)	1:1000	+++	+++	++	+	ND	−	ND	Chemicon	[29]
MM13-4	MC	N-term (25–35)	1:1000	+++	+++	+++	+	ND	−	ND	Chemicon	[29]
CC24-R	PC	R domain (693–716)	1:2000	+++	+++	++	+	ND	ND	ND	H de Jonge	[26]
R3195	PC	C-term	1:3000	++	+++	+++	+	ND	−	ND	C Marino	[28]
MP-CT1	PC	C-term	1:2000	ND	+++	+++	++	ND	−	ND	R Dormer	[27]
LIS-1	PC/S	C-term (1468–1480)	1:200	+	+++	ND	ND	+	ND	ND	Our lab	–
MATG 1104	MC	R-dom (722–734)	1:500	++	+++	+	−	ND	ND	ND	Transgène	–
NBD1-Birm	PC	NBD1	1:2000	++	+++	+++	+	+	ND	+	D Cyr	–
181	PC	Pre-NBD_1 (415–427)	1:1000	Unsp	Unsp	Unsp	Unsp	Unsp	ND	Unsp	W Guggino	[24]
GA-1	MC	NBD2-C-term	1:500	−	−	−	−	ND	−	ND	K Kirk	–
C1468	PC	C-term (1468–1480)	1:500	−	−	ND	−	−	ND	−	R Kopito	[30]

Results from other applications (e.g. immunocytochemistry) are not mentioned here, see for instance, Ref. [9].
[a] MC, monoclonal; PC, polyclonal; S, non-purified serum.
[b] +++, Good detection;++, reasonable detection; +, poor detection; −, no detection; Unsp, unspecific; ND: not done.
[c] With either wt- or F508del-CFTR.

aa residues 2–79 [25], AbNBD1-Birmingham, generated against a CFTR–GST fusion protein corresponding to the NBD1 (from D. Cyr, Birmingham, AL, USA), AbCC24-R, generated against aa residues 693–716 [26] MP-CT1, generated against the C-terminus of CFTR [27], and AbR3195, generated against aa residues 1468–1480 [28]. The following mouse monoclonal anti-CFTR antibodies (mAbs) were used (see also Fig. 1 and Tables 1 and 2): AbM3A7, generated against aa residues 1197–1480 [29] from *Chemicon* (Temecula, CA, USA) (1 mg/ml), AbL12B4, generated against aa residues 386–412 [29] from *Chemicon* (1 mg/ml), AbMM13-4, generated against aa residues 24–35 [29] from *Chemicon* (1 mg/ml), AbGA-1, generated against the C-terminus of CFTR (from K. Kirk, Birmingham, AL, USA), and AbMATG 1104, generated against aa residues 722–734 of CFTR (from *Transgène*, Strasbourg, France). All Abs obtained from research laboratories were affinity-purified by each group and were

Table 2
Applicability of different antibodies for the biochemical detection of CFTR by immunoprecipitation

Ab	Type[a]	Epitope	Dilution	Applicability[b]					Origin	Ref.
				CFTR transfected[c]		Endogenously expressing CFTR				
				CHO	BHK	Calu-3	16HBE	IB3-1		
M3A7	MC	C-term (1197–1480)	1:500	+++	+++	+	++	ND	Chemicon	[29]
L12B4	MC	Pre-NBD1 (386–412)	1:400	ND	+++	ND	−	−	Chemicon	[29]
MM13-4	MC	N-term (25–35)	1:400	+++	+++	+	+	−	Chemicon	[29]
CC24-R	PC	R domain (693–716)	1:200	ND	+	−	−	−	H de Jonge	[26]
R3195	PC	C-term	1:500	ND	+++	ND	ND	ND	C Marino	[28]
MP-CT1	PC	C-term	1:10	ND	+++	−	+	ND	R Dormer	[27]
LIS-1	PC/S	C-term (1468–1480)	1:50	+	+++	ND	ND	ND	Our lab	−
GA-1	MC	NBD2-C-term	1:200	ND	+++	−	+	−	K Kirk	−
N-term-Birm	PC	N-term (2–79)	1:200	+++	ND	ND	ND	ND	D Cyr	[25]
NBD1-Birm	PC	NBD1	1:200	+++	ND	ND	+	ND	D Cyr	–
169	PC	R-dom (724–746)	1:200	+++	+++	+	+	ND	W Guggino	[24]
C1468	PC	C-term (1468–1480)	1:100	−	ND	ND	ND	ND	R Kopito	[30]
MATG 1104	MC	R-dom (722–734)	1:250	−	ND	ND	−	ND	Transgène	–

[a] MC, monoclonal; PC, polyclonal; S, non-purified serum.
[b] +++, good detection; ++, reasonable detection; +, poor detection; −, no detection; Unsp, unspecific; ND, not done.
[c] With either wt- or F508del-CFTR.

used at concentrations recommended (and in most cases, published) by their providers as optimal for either WB or IP. Often experimental conditions used here did not correspond exactly to the original recommendations but were the same for all the Abs tested in order to allow for a better comparison. pAb C1468, generated against aa residues 1468–1480 [30], was also tested in our experiments but results are not shown because they were always negative (i.e. no bands were detected).

The secondary horseradish peroxidase-conjugated antibodies (sAbs) anti-mouse IgG and anti-rabbit IgG (*Amersham*, Piscataway, CA, USA) were used for the detection of primary anti-CFTR mAbs and pAbs, respectively.

2.4. Western blot

For Western blot (WB) analyses, about 2×10^6 cells were lysed with sample buffer (1.5% (w/v) SDS, 5% (v/v) glycerol, 0.001% (w/v) bromophenol blue, 0.5 mM dithiotreitol, and 31.25 mM Tris pH 6.8). DNA was sheared by passing the sample first through a 22G and then a 27G needle until the viscosity dropped. Total protein was quantified by a modified micro Lowry method and aliquots of 30 μg of total protein were transferred onto nitrocellulose filters (*Schleicher and Schuell*, Dassel, Germany) after electrophoretic separation on 7% (w/v) polyacrylamide mini-gels (*BioRad* Mini-Protean II System). The filters were probed with each of the anti-CFTR Abs indicated above in 5% (w/v) skim milk, at dilutions mentioned in Table 1, overnight at 4 °C. After three 5-min washes with PBS at room temperature (RT), filters were incubated for 1 h with the respective sAb, diluted 1:3000 at RT, again washed three times with PBS and developed using the ECL™ detection system (*Amersham*). Blots were reprobed with an anti-actin antibody (A-4700, *Sigma*, St Louis, Missouri) as an internal control (data not shown). Experiments were repeated at least three times. Only results obtained consistently, and for which reproducibility was clear, are shown.

2.5. [^{35}S] Labelling and immunoprecipitation of CFTR

Sub-confluent P60 dishes were washed twice with 2 ml of Hank's balanced salt solution (*Life Technologies*, CA, USA) and incubated in methionine-free medium for 30 min. Labelling was performed with 1 ml methionine-free medium containing 140 μCi/ml [^{35}S] methionine (Tran^{35}S-Label reagent, *ICN*, Costa Mesa, CA, USA, 10,5 mCi/ml) for 2 h. Cells were lysed in 1 ml RIPA buffer (1% (w/v) deoxycolic acid, 1% (v/v) Triton X-100; 0.1% (w/v) SDS, 50 mM Tris pH 7.4, 150 mM NaCl). The IP was carried out as described elsewhere [31]. Briefly, incubation with an appropriate dilution of each Ab (as indicated in Table 1) was carried out overnight at 4 °C with mild shaking, followed by a 4 h-incubation with protein A beads for pAbs or protein G beads for mAbs (*Roche*). Immunoprecipitated proteins were eluted from the beads with sample buffer (see above) for 1 h at RT and then electrophoretically separated on 7% (w/v) polyacrylamide gels (*Hoefer* SE 400). For fluorography, gels were pre-fixed in methanol/acetic acid (30:10, v/v), rinsed with water and soaked into 1 M sodium salicylate for 60 min. After drying at 80 °C for 2 h, gels were exposed to Biomax X-ray films (*Kodak*, Rochester, NY, USA) for 24 h. Results shown were repeatedly obtained as described for Westerns.

3. Results

3.1. Western blot

Fig. 2 shows results obtained by WB for non-transfected BHK cells (lane 1), BHK cells stably expressing either wt- (lane 2) or F508del-CFTR (lane 3), Calu-3 cells (lane 4) and 16HBE14o-cells (lane 5) with several anti-CFTR Abs, namely, M3A7 mAb (A), L12B4 mAb (B), MM13-4 mAb (C), CC24-R pAb (D), R3195 pAb (E), MP-CT1 pAb (F), Lis-1 pAb (G), MATG1104 mAb (H) and NBD1-Birmingham pAb (I). wt-CFTR was detected with M3A7 mAb, both in respiratory cell lines endogenously expressing it such

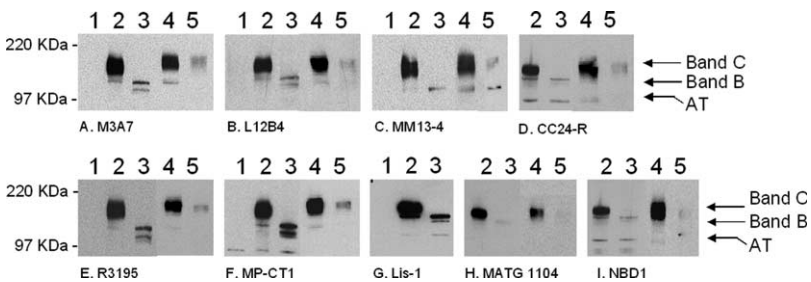

Fig. 2. Detection of CFTR by WB with the following Abs: (A) M3A7 mAb, (B) L12B4 mAb, (C) MM13-4 mAb, (D) CC24R pAb, (E) R3195 pAb, (F) MP-CT1 pAb, (G) Lis-1 pAb, (H) MATG1104 mAb and (I) NBD-1 pAb. Thirty micrograms of total protein were applied per lane. Results are from non-transfected BHK cells (lane 1), BHK cells stably expressing either wt- (lane 2) or F508del-CFTR (lane 3) and from endogenously CFTR-expressing Calu-3 cells (lane 4) and 16HBE14o-cells (lane 5). The positions of 97 and 220 kDa molecular mass markers are shown. Arrows indicate the relative positions of the ER core-glycosylated (band B), the post-Golgi fully-glycosylated (band C) forms of CFTR and of an extra-band, plausibly resulting from alternative translation initiation (AT) product. Results with MATG1104 mAb were obtained after treatment with 4-PBA (Section 2).

as Calu-3 (Fig. 2A, lane 4), 16HBE14o- (Fig. 2A, lane 5) or the colonic cell line HT29 (not shown) and in the wt-CFTR stably transfected BHK cells (Fig. 2A, lane 2). In all these cell lines, both the 150 kDa band, corresponding to the core-glycosylated (band B), and the 170 kDa band, corresponding to fully-glycosylated CFTR (band C), could be detected (see arrows on the right of panels). No significant differences in CFTR expression by 16HBE14o- were found when non and polarized cells were compared. M3A7 was considered as the reference Ab in subsequent biochemical analyses. wt-CFTR expressed in stably transfected BHK cells (always lane 2 in Fig. 2) was also successfully and clearly detected by WB with the following Abs (see also Table 1): L12B4 (panel B), MM13-4 (C), CC24-R (D), R3195 (E), MP-CT1 (F), Lis-1 (G), MATG1104 (but only after 4-PBA treatment (H)) and NBD1-Birmingham (I). wt-CFTR endogenously expressed by Calu-3 cells (always lane 4 in Fig. 2) was detected by WB with Abs (Table 1): L12B4 (panel B), MM13-4 (C), CC24-R (D), R3195 (E), MP-CT1 (F), MATG1104 (H) and NBD1-Birmingham (I). wt-CFTR endogenously expressed in 16HBE14o- cells (always lane 5 in Fig. 2) was detected by WB with Abs (Table 1): L12B4 (panel B), MM13-4 (C), CC24-R (D), R3195 (E), MP-CT1 (F) and NBD1-Birmingham (I) but not with Ab MATG1104 (H). Similarly, F508del-CFTR expressed in stably transfected BHK cells (always lane 3 in Fig. 2) was successfully detected by WB with Abs (Table 1): L12B4 (panel B), MM13-4 (C), CC24R (D), R3195 (E), MP-CT1 (F), Lis-1 (G), MATG1104 only after 4-PBA treatment and even in these conditions at low levels (H) and NBD-1 Birmingham (I). F508del-CFTR was also detected by WB with the M3A7 mAb in stably transfected CHO and BHK cells, but only as the core-glycosylated band B (Fig. 2A, lane 3 and Table 1). CFTR was not detected in non-transfected BHK cells (Fig. 2A, lane 1) or in F508del/W1282X IB3-1 cells (not shown). In WB experiments, a band of lower molecular mass than band B (marked AT) was detected in BHK cells stably expressing F508del-CFTR (lane 3) by several Abs, namely; M3A7 (A), L12B4 (B), R3195 (E), MP-CT1 (F) and Lis-1 (G). This band was previously shown to correspond to a polypeptide resulting from usage of an alternative translation initiation site of the CFTR mRNA [32]. One of the most interesting differences observed from WB results was the failure of the MM13-4 mAb, raised against N-terminus of CFTR, to detect a band of lower molecular mass than band B. This supports the conclusion [32] that this lower band corresponds to a protein that results from use of an alternative translation start site in the CFTR mRNA.

Table 1 summarizes WB results obtained in the various cell lines with all Abs tested in the present study. A relative scale was adopted, ranging from '+++' (very good detection, e.g. detection of wt-CFTR by M3A7 in BHK cells stably expressing wt-CFTR) to ' − ' (no detection). With the Abs GA-1 (five cell lines tested), 181 (five cell lines tested) and C1468 (five cell lines tested), we were not able to detect CFTR in any of the cell lines either due to absence of bands in the area of molecular mass where CFTR should appear (GA-1 and C1468) or due to the appearance of too many non-specific bands (181 pAb) that did not differ from the negative controls (data not shown). Although unlikely, we cannot rule out that the aliquots of the Abs that we used did not undergo changes (e.g. degradation) since they were originally produced.

3.2. Immunoprecipitation

Fig. 3 summarizes results obtained by IP for non-transfected BHK cells (lane 1), BHK cells stably expressing either wt- (lane 2) or F508del-CFTR (lane 3), Calu-3 cells (lane 4) and 16HBE14o-cells (lane 5). wt-CFTR was detected by IP with the M3A7 mAb in the endogenously expressing cell lines, Calu-3 (Fig. 3A, lane 4), 16HBE14o- (albeit at low levels; Fig. 3A, lane 5) and HT29 (not shown), and in stably transfected BHK cells (Fig. 3A, lane 2). In all these cell lines, M3A7 detected both bands B and C (see arrows on the right of panels). F508del-CFTR was also detected by IP with M3A7 in stably transfected CHO and BHK cells but, as in WB, only as band B (Fig. 3A, lane 3 and Table 1). CFTR was not detected by IP in non-transfected BHK cells (Fig. 3A, lane 1), nor in F508del/W1282X IB3-1 cells (not shown). As in WB, IP analyses

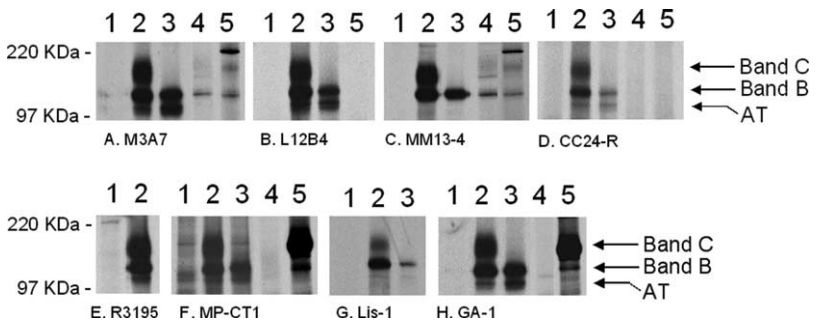

Fig. 3. Detection of CFTR by [^{35}S] labelling and IP with the following Abs: (A) M3A7 mAb, (B) L12B4 mAb, (C) MM13-4 mAb, (D) CC24-R pAb, (E) R3195 mAb, (F) MP-CT1 pAb, (G) Lis-1 pAb and (H) GA-1 mAb. IPs were performed from sub-confluent P60 dishes. Results are from non-transfected BHK cells (lane 1), BHK cells stably expressing either wt- (lane 2) or F508del-CFTR (lane 3) and endogenously CFTR-expressing Calu-3 (lane 4) and 16HBE14o-cells (lane 5). Molecular mass markers and arrows as in the legend of Fig. 2.

detected the band (marked AT) corresponding to the alternative translation initiation site with all Abs, except for MM13-4, which is N-terminal specific (Fig. 3C) and N-term-Birmingham (not shown). Results obtained for IP with the M3A7 were also considered as reference in IP analyses with other Abs. wt-CFTR expressed by stably transfected BHK cells (always lane 2 in Fig. 3) was detected by IP with all Abs tested, namely: L12B4 (panel B), MM13-4 (C), CC24-R (D), R3195 (E), MP-CT1 (F), Lis-1 (G) and GA-1 (H) and N-term-Birmingham mAb (not shown). wt-CFTR endogenously expressed by Calu-3 cells (always lane 4 in Fig. 3) was detected by IP with Ab MM13-4 (panel C), but not with Abs CC24-R (D), MP-CT1 (F) nor GA-1 (H). wt-CFTR endogenously expressed by 16HBE14o- cells (always lane 5 in Fig. 3) was detected by IP with the following Abs: MM13-4 (panel C), MP-CT1 (F) and GA-1 (H), but not with L12B4 (B) nor CC24-R (D) Abs. Finally, F508del-CFTR expressed by stably transfected BHK cells (always lane 3 in Fig. 3) was detected by IP with all Abs tested, namely: L12B4 (panel B), MM13-4 (C), CC24-R (D), MP-CT1 (F), Lis-1 (G) and GA-1 (H) and with N-term-Birmingham mAb.

Table 2 summarizes CFTR IP results obtained for all cell lines and Abs tested in this study. The relative scale adopted is the same as for Table 1.

4. Discussion

Biochemical detection of CFTR protein has been a matter of considerable controversy and only recently have there been commercially available Abs that produce clear and unequivocal results. One of the major constraints for the biochemical analysis of CFTR in native cells is its low abundance. Abs with high specificity and sensitivity would be valuable tools in the diagnosis and monitoring of CF, especially to monitor novel therapies for CF acting at the protein level [33]. Here, we detected CFTR endogenously expressed by human epithelial cells such as the respiratory lines Calu-3 [34] and 16HBE14o- [35]. Less surprising is the successful detection of CFTR in stably transfected cell lines like CHO or BHK. Results, however, depend not only on the cell line analysed, but also on the Ab used. For example, we show that CFTR can be detected by WB in 16HBE14o-cells with M3A7 but not with MATG1104 or in Calu-3 cells by IP with M3A7 but not with MP-CT1.

Some of the anti-CFTR Abs we tested were found to be nonspecific in our hands. This was the case of the Abs 181 and MP-CT1. Based on our data we strongly recommend that both positive and negative controls should always be analysed together with experimental samples. The absence of bands in cell lines that do not express CFTR reinforces the certainty that bands in test samples of the right size do, indeed, correspond to CFTR. Likewise, the use of cells expressing detectable levels of CFTR is required as a positive CFTR reference. Cell lines stably expressing wt-CFTR are useful as controls to detect both band B and band C, whereas cells stably expressing solely F508del-CFTR are useful as band-B positive, and band-C negative controls.

Fully-glycosylated CFTR is characteristically detected as a diffuse band, or even as a complex pattern of bands, in the vicinity of 170 kDa. If a single sharp band in the vicinity of 170 kDa is detected, it is very unlikely that it is band C of CFTR. Additionally, to confirm that a detected band is fully glycosylated CFTR, tests with specific glycosydases, such as endoglycosidase H and N-glycosidase, should be performed. The same test should be applied to confirm the identity of a band as the immature form of CFTR (band B). In mammalian cells, CFTR mRNA has been reported to produce two protein products resulting from alternative initiation sites. These have been designated as ‘double band B’ [32]. Consistent with this explanation are results shown here with the Abs specific for the N-terminal of the protein (MM13-4 Abs, Figs. 2 and 3—panel C, and N-term-Birmingham Abs, Figs. 2 and 3—panel C) that fail to detect the lower of the two bands in double band B. However, confirmation of this hypothesis may require characterization by mass spectrometry. CFTR, when overexpressed, tends to form aggregates [30,36] and this phenomenon may lead to the appearance of extra bands of higher molecular mass if adequate sample solubilization is not carried out following lysis. Similarly, higher molecular mass bands corresponding to either ubiquitinated forms may also appear [37] as, for instance, in Fig. 3 lane 5 in panels A and C. On the other hand, lower molecular mass bands may correspond to CFTR forms resulting from proteolytic cleavage. It is possible to partially overcome these events from occuring in vitro by using cocktails of protease inhibitors in the lysis buffer. The use of a particular Ab always raises the issue of specificity. No Abs are monospecific, including mAbs. If a pAb is being used, however, it is more likely to produce cross-reactivity than a mAb. A given Ab may appear as monospecific for a certain cell type but when used on a different cell line or tissue, cross-reactivity may occur. Although the appearance of bands with the right size in immunoblots is generally considered as good practice to control Ab specificity, this is not final proof of protein identification and neither is peptide-competition for Ab binding. Indeed, in the latter case, cross-reactive proteins may have short peptide sequences that are homologous to the target epitope and thus peptide competition will also abolish the cross-reaction signal. The only true proof of protein identity is characterization by definitive methods like peptide sequencing or mass spectrometry.

Results obtained with different Abs are also dependent on how the Ab is used. For example, CFTR from Calu-3 cells is, in general, better detected by WB than by IP. WB detects total levels of a protein at steady state while the IP protocol used here is preceded by a radioactive labelling step (to avoid detection of Ig chains) so only newly synthesized CFTR is observed. In cells like Calu-3, where CFTR is

relatively stable, there may be little new synthesis occurring and IP is less sensitive, unless labelling is performed for long periods of time. Likewise, the mAb MATG1104 detects CFTR by WB but does not significantly, immunoprecipitate the protein. Also, mAb GA-1, in our hands, succeeded in immunoprecipitating CFTR but failed to detect it by WB.

Abs tested here that showed the best results for the detection of wt- and F508del-CFTR by WB both in stably transfected (BHK or CHO) and in endogenously expressing cell lines (Calu-3 or 16HBE14o-) were M3A7, L12B4, MM13-4, NBD1-Birmingham, MATG 1104, Lis-1, MP-CT1, R3195, CC24-R, N-term-Birmingham. Abs 181, C1468, and GA-1 did not produce good results for WB analysis of CFTR in our hands, due to the presence of many non-specific bands or due to the total absence of bands. Abs that showed the best results for the detection of wt- and F508del-CFTR by IP in both stably transfected (CHO or BHK) and in endogenously expressing cell lines (Calu-3 or 16HBE14o-) were 169, N-term-Birmingham, NBD1-Birmingham, M3A7, Lis-1, L12B4, MM13-4, MP-CT1, GA-1, R3195 and MP-CT1. The MATG1104 and C1468 Abs did not produce useful results in IPs for either wt- or F508del-CFTR due to either the presence of non-specific bands or to the total absence of bands, respectively.

Acknowledgements

Antibodies. H. de Jonge, Rotterdam, Netherlands (CC24-R), K. Kirk, Birmingham, AL, USA (GA-1), C. Marino, Memphis, TN, USA (R3195), B. Dormer, Cardiff, UK (MP-CT1), Transgène, Strasbourg, France (MATG 1104), J Riordan, Scottsdale, AZ, USA (M3A7), D. Cyr, Birmingham, AL, USA (N-term-Birmingham and NBD1-Birmingham), W.B. Guggino, Baltimore, MD, USA (169 and 181), R. Kopito, Stanford, CA, USA (C1468). *Cells*. G. Lukacs, Toronto, Canada (BHK stably transf.), G. Cutting, Baltimore, MD, USA (IB3-1), M. Hug, Freiburg, Germany (Calu-3), J. Riordan, Scottsdale, AZ, USA (CHO stably transf.), D. Gruenert, Colchester, VT, USA (16HBE14o- and ΣCFTE29o-). *Funding*. POCTI/MGI/35737/99 research grant; EU CF Network QLK-1999-00241; Praxis XXI BD/11094/97 (CMF), BD/19869/99 (MRR) and BD/21440/99 (FM) fellowships.

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