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Biochemical methods to assess CFTR expression and membrane localization

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Abstract

Detection of cystic fibrosis transmembrane conductance regulator (CFTR) protein is usually a difficult task to accomplish due to the low levels of expression and high turnover that this membrane protein is submitted to in the cell. Common biochemical methods can be used for the detection of CFTR but several critical points must be taken into account. The scope of this article is to outline biochemical methods commonly used to assess CFTR expression, processing and membrane localization.

Keywords: CFTR; ABC transporter; ABCC7; Biochemical analyses; Methods

1. Introduction

Cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein, product of the CF gene, 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, a member of the ATP-binding cassette (ABC) transporter superfamily (ABCC7), has 1480 aminoacid residues and comprises several domains: two transmembrane domains (TMs 1 and 2, each one with six membrane spanning segments), two nucleotide binding domains (NBDs 1 and 2) and a large regulatory (R) domain [1].

Like other glycoproteins, CFTR is synthesized on endoplasmic reticulum (ER)-associated ribosomes and core-oligosaccharide chains are attached to the protein co-translationally at the asparagines (N) residues 894 and 900 located in the 4th extracellular loop [2]. A large proportion of CFTR is degraded from the ER. The remaining are

Abbreviations: Ab, antibody; AP, alkaline phosphatase; ABC, ATP-binding cassette (transporter); DAB, 3,3-diaminobenzidine tetrahydrochloride; DOC, sodium deoxycholate; DTT, dithiotreitol; ELISA, enzyme-linked immunosorbant assay; Endo H, endoglycosidase H; ER, endoplasmic reticulum; HRP, horseradish peroxidase; IP, immunoprecipitation; NP-40, nonidet P-40; PAGE, polyacrylamide gel electrophoresis; 4-PB, 4-phenyl butyrate; PBS, phosphate-buffered saline; PNGase F, *N*-glycosidase F; R, (domain) regulatory (domain); SDS, sodium dodecyl sulphate; TM, transmembrane domain; WB, Western blot.

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transported to the Golgi. Along the secretory pathway, the polypeptidic chain undergoes different post-translational modifications at its glycidic residues to produce the fully glycosylated mature form. Monitoring the glycosylation state of CFTR is thus a convenient way to follow the intracellular processing of CFTR, and it allows to distinguish the wild-type (wt) protein from the several processing mutants. According to its glycosylation status, one can distinguish three diferent forms of the CFTR: (1) the newly synthesized non-glycosylated polypeptidic chain (only biochemically detected following treatment with glycosidases), known as band A (130 kDa); (2) the ER core-glycosylated form of the protein, known as band B (150 kDa); and (3) the fully glycosylated mature form of CFTR, known as band C (170–180 kDa) [3].

Soon after cloning of the CF gene in 1989 [4–6] and prediction of the respective protein structure [4], great interest arouse in its biochemical detection by Western blot (WB) or by immunoprecipitation (IP). However, this is not an easy task due to its very low levels of expression in native tissues and low sensitivity/specificity of anti-CFTR antibodies (Abs) (see Ref. [7]). Since the design of the experimental protocol to biochemically detect CFTR expression is also critical, we review here these protocols (with the details being given elsewhere [8]).

2. Methods to assess CFTR expression

Generally, the biochemical methods of WB and IP aim at determining the presence of a given protein in a sample under study, or to specifically determine its relative abundance or molecular mass (and hence, its glycosylation status and subcellular localization). The latter is particularly important to assess the effect of mutations on processing, which is generally carried out in heterologous expression systems, due to the low levels of endogenous expression in most tissues. Production of a mutant cDNA cloned into an mammalian expression vector is thus a valuable tool to analyze properties of CFTR mutants, namely in terms of folding, structure and function.

2.1. Site-directed mutagenesis on CFTR-cDNA

For the rapid introduction of point mutations, small deletions/insertions into a sequence (i.e., CFTR cDNA cloned in a given expression vector) there is a commercially available site-directed mutagenesis technique (QuikChange®, Catalog #200518/9; Stratagene, La Jolla, CA, USA) that is easy to perform and a high success rate. The detailed protocol is detailed elsewhere [9]. The method is generally based on the use of two complementary oligonucleotide primers of 25–45 nucleotides, containing the desired mutation in the middle region, flanked by unmodified nucleotide sequences. *Pfu Turbo* DNA polymerase, under specific cycling parameters, extends these primers in both directions and generates, with high fidelity, a mutant plasmid, although still containing nicks. To check for the mutagenesis efficiency, the amplified product is analyzed on agarose gel, following hydrolysis with *Dpn*I, which is specific for methylated and hemi-methylated DNA thus removing the parental plasmid. The nicked mutant plasmid is used to transform competent *Escherichia coli* cells that efficiently repair the nicks and amplify it. The presence of the desired mutation should be confirmed by sequencing. The mutant CFTR cDNA plasmid, may be subsequently used to transiently or stably transfect mammalian cells lines which will thus produce the mutant protein.

2.2. Detection of CFTR protein expression in human and mouse tissues and in cultured cells by Western blotting

The detailed protocol of WB is described in detail elsewhere [8]. It can be used to check the presence of CFTR protein or its relative abundance and molecular mass.

In general, WB is robust, simple and sensitive. However, it is elusive when applied to the detection of CFTR in native tissues, due to the low abundance of this protein. Other reasons account for the unsatisfactory biochemical detection of CFTR in cell lines, like the frequent lack of sensitivity and specificity of anti-CFTR Abs [7]. The outlined WB protocol [8] is simple and reproducible when applied to samples of cultured cells. For native tissues (human or mouse), however, additional manipulations are recommended, including isolation of membranes by differential centrifugation to increase CFTR concentration and incubation of samples in excess cold saline and a cocktail of protease inhibitors, prior to protein extraction.

WB is composed of five main steps: (1) preparation of CFTR sample (cell lysis); (2) resolution by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE); (3) transfer of separated proteins onto a membrane (nitrocellulose or PVDF); (4) staining the blot to assess efficiency of protein transfer (can be omitted); and (5) CFTR detection with a specific antibody (Ab).

After washing cells with PBS, lysis is performed in gel electrophoresis sample buffer (containing 5% (w/v) SDS and 0.5 mM DTT). It is very important to avoid boiling the samples. Instead, incubation at 55 °C for 15 min is carried out. Chromosomal DNA is sheared to lower viscosity by passing the sample sequentially through 22- and 27-gauge (ga.) needles. After protein quantification (Lowry), about 30–50 μg (depending on expression levels of cell line under analysis) of total protein per sample are separated by SDS-PAGE on a 7% (w/v) polyacrylamide resolving gel.

After electrophoresis, a common wet-transfer electroblotting procedure is carried out. Prior to CFTR detection, the membrane is blocked (5% (w/v) skimmed dry milk; 0.1% (v/v) Tween 20 in PBS) to prevent non-specific adsorption of the Abs. Indirect detection is performed by incubation with primary anti-CFTR Ab, and then with a labelled (e.g., with horseradish peroxidase, HRP) secondary

Ab. Detection on X-ray film is then performed by a chemiluminescence approach. Alternatively, an alkaline phosphatase (AP)-conjugated secondary Ab may be used and detection is performed with 3,3-diaminobenzidine tetrahydrochloride (DAB).

Immunostaining specificity can be confirmed by adequate controls and purified Abs. Polyclonal anti-CFTR Abs raised against rodent or human CFTR peptides should be affinity-purified [7] and ideally samples from CFTR-null mice (or patients with two stop mutations) should be included. Formation of high molecular mass aggregates is minimized if lysis is performed for a longer period (30 min) at room temperature (RT).

2.3. CFTR immunoprecipitation

The complete protocol of IP [8] consists in partial isolation of CFTR protein, from total lysates. IP can be used to determine the molecular mass, to study protein/protein associations [10], monitor the appearance of co/post-translational modifications and, when coupled to a pulse-chase protocol, to determine the protein half-life.

The IP protocol comprises three different steps: (1) preparation of cell lysate; (2) pre-clearing the lysate of non-specific background (this step can be omitted); and (3) formation and purification of the antigen–Ab complex.

The lysis method depends on the cell type under study and the final experimental purpose. No single buffer has applicability to all purposes. In general, lysis conditions should be as gentle as possible to retain the epitope(s) to be recognized. The most common cell lysis buffer is RIPA. It releases most soluble cytoplasmic and nuclear proteins, but also disrupts most protein–protein interactions.

Pre-clearing consists in removal of proteins non-specifically bound to the immune complex or to protein A (or G) beads prior to IP. Pre-clearing can be omitted if non-specific interactions are not expected in large extent or if, as for CFTR, in vitro degradation of the target protein (and thus time) is critical.

Theoretically, the Ab–CFTR complex formation is the simplest step in the protocol. The Ab is added to the lysate binding to CFTR present. Then protein A (or G) beads are added. After mixing and incubation for ~4 h at 4 °C, proteins not bound to the beads are washed out with lysis buffer. The Ab amount used is critical, depending on the Ab itself (its avidity) and on the titre of the serum or ascites fluid. Recommended dilutions for anti-CFTR Abs are reviewed elsewhere [7]. The IP reaction volume is also important, if too small, unspecific binding occurs and if too large, CFTR-Ab binding becomes excessively slow.

Elution of Ab-bound proteins is also critical, as drastic procedures like heating or shaking will diminish the final yield. Elution can be performed by Laemmli application buffer (“cracking buffer”) that includes SDS, a reducing agent [dithiotreitol (DTT) or β-mercaptoethanol] and a component to increase sample density for gel loading (e.g., glycerol or sucrose).

After gel electrophoresis, detection of CFTR is performed by Coomassie or silver staining (see Ref. [11]) if the objective is simply the detection (presence/absence) of specific CFTR processing forms (bands B and C). To increase sensitivity, cells can be radio-labelled by, e.g., [^{35}S]methionine and/or [^{35}S]cysteine and IP performed as above.

Radio-labelling can also used for the determination of CFTR half-life by a pulse-chase performed prior to IP. The radioactive aminoacid is added to the medium for, e.g., 20 min (‘pulse’), to be incorporated into nascent proteins. The radioactive medium is then replaced by another with excess of unlabelled aminoacid. IPs are then performed at different times (‘chase’) after medium replacement (e.g., 0, 0.5, 1, 2 and 3 h). The determined rate of radioactivity loss is a measure of CFTR turnover.

WB detection of immunoprecipitated CFTR or phosphorylation (with ^{32}P) of CFTR prior to elution can be used for enhanced IP sensitivity. The latter is to be used mainly for accurate quantification of steady-state level of bands B and C. It makes use of the presence of several consensus phosphorylation sites for cAMP-dependent protein kinase A (PKA) in the R domain. However, the level of in vivo phosphorylation affects the results.

3. Detection of CFTR expression at the plasma membrane

Cell-surface biotinylation is useful to distinguish cell surface from intracellular proteins, thus, allowing to study CFTR expression exclusively at the plasma membrane. A non-cleavable form of biotin reagent (EZ-Link Biotin-LC-hydrazide; Pierce Rockford, IL, USA) is chosen over cleavable biotin reagent (e.g., EZ-Link Sulfo-NHS-S-S-biotin, Pierce), mainly for two reasons: (a) Biotin-LC-hydrazide has binding affinity to carbohydrates present on the 4th extracellular loop of CFTR (see Introduction), and (b) disulfide bonds present in Sulfo-NHS-S-S-biotin (which has affinity for amino groups) cannot be cleaved by reducing agents as efficiently as claimed by the manufacturer. This may lead to contamination of biotinylated (surface) proteins with the unbiotinylated (intracellular) proteins. However, unlike Sulfo-NHS-S-S-biotin, Biotin-LC-hydrazide is cell permeable. Thus, out the biotinylation protocol should be carried out on ice (with ice-cold buffer) to prevent its intracellular incorporation. Since Biotin-LC-hydrazide is non-cleavable, WB only allows indirect measurement of surface expressed CFTR.

Cells stably expressing (wt or mutant) CFTR [11] are grown to 70% confluency and then either directly analysed or treated with 5 mM 4-phenyl sodium butyrate (4-PB) for 24–60 h to up-regulate CFTR expression [12,13]. Cells expressing processing mutants, like F508del, should be incubated with 4-PB at 27 °C to induce surface expression

[12,13]. The protocol is described in detail at the European Working Group on CFTR Expression website [8]. Briefly, cell monolayers are washed 3 × with PBS/c-m (i.e., PBS with 0.1 mM $CaCl_2 \cdot 2H_2O$; 1 mM $MgCl_2$) and incubated for 30 min with periodate solution (10 mM $NaIO_4$ in PBS/c-m) to oxidize the carbohydrate moieties on cell surface. Cells are washed again as above, followed by a single wash with acetate buffer (100 mM sodium acetate; 0.1 mM $CaCl_2 \cdot 2H_2O$; 1 mM $MgCl_2$). All steps are carried out on ice and in the dark with ice-cold buffer, unless otherwise stated. Cells are incubated with 2 mM biotin-LC-hydrazide for 30 min. Unbound biotin is removed by three PBS-c/m washes. Cell lysates are prepared as above for WB [8]. Biotinylated (surface) proteins are irreversibly extracted with streptavidin immobilized on 4% beaded agarose (SA). Simultaneously, an aliquot of lysate is also processed with uncoated control agarose beads (CN). Supernatants from SA beads contain only unbiotinylated (intracellular) proteins, whereas supernatants from CN beads contain both surface and intracellular proteins.

Thirty micrograms of total protein supernatants from SA or CN beads are analysed by WB (see above). The scanned CFTR signal from SA beads is subtracted from CN beads signal to indirectly determine the surface expressed CFTR.

It is essential that SA beads completely extract the biotinylated proteins to avoid any contamination of unbiotinyalted (intracellular) proteins with that of biotinylated (surface) proteins. Following WB the membrane is stripped off of the Ab [8] and incubated with streptavidin–HRP (4 μg conjugate/ml). Colour is developed by DAB and the reaction stopped by several washes with water. When efficiently extracted, the SA beads sample does not evidence any bands, whereas the CN beads sample evidences a number of bands, i.e., all biotinylated proteins [12,13]. Biotinylated proteins can also be visualized immediately after protein transfer to nylon membrane (see above).

An enzyme-linked immunosorbant assay (ELISA) variant of this biotinylation protocol has also been described for CFTR [8]. This has the major advantage of being performed in SA-coated 96-well micro plates, thus allowing the rapid screening of, e.g., on CFTR processing mutants. A detailed protocol is found elsewhere[8]. Briefly, cells are surface biotinylated and lysates immunoprecipitated using one anti-CFTR Ab. Biotinylated-CFTR then binds to streptravidin-coated ELISA plates and detected by one anti-CFTR Ab and AP-or HRP-conjugated secondary Ab. Colour is developed by adding a substrate of HRP specially designed for ELISA (e.g., ABTS® from Roche), and spectrophotometically read at 405 nm. Controls include assays where primary or secondary Abs are omitted.

4. Membrane preparation and CFTR deglycosylation

The isolation of microsomal membrane vesicles from BHK-21 cells stably expressing CFTR is based on a protocol developed earlier for CHO cell fractionation [14]. The protocol discussed here is described elsewhere in detail [8] and it is similar to that employed for CFTR channel gating studies in lipid bilayers [15,16]. CFTR is enriched in membranes compared to cell lysates, therefore it is easier to detect (by IP or WB) in membrane preparations. These have been used in many applications, where increased amounts of CFTR, but not the purified protein, are required, such as photo-labelling of CFTR by 8-azido-ATP [17]. Furthermore, membrane vesicles are commonly utilized for the characterization of wt and mutant CFTR in single-channel measurements [16,18,19].

The difference in molecular mass between fully complex glycosylated and core-glycosylated protein can be detected by WB, as described above. However, final proof of the gycosylation status of CFTR can be obtained by tests with specific glycosidases. Peptide *N*-Glycosidase F (PNGase F, *N*-Glycanase) deglycosylates core-and complex-glycosylated CFTR completely. Endoglycosidase H (Endo H) only removes unprocessed core-oligosaccharide chains.

The preparation of microsomal membranes followed by enzymatic deglycosylation is a straightforward procedure to identify the glycosylation state of CFTR. The following precautions should be considered to obtain good results. Before starting the membrane preparation, cell culture dishes should be placed on ice and kept there (or at 4 °C) during all subsequent steps. The homogenization of the microsomal pellet is facilitated by brief sonication.

Prior to deglycosylation, proteins are usually denaturated by SDS at 95 °C. However, like other integral membrane proteins, CFTR has a tendency to form aggregates upon boiling and therefore samples should not be treated this way (see above, WB protocol). CFTR is also very sensitive to proteolytic degradation, thus use of protease-free deglycosylation enzymes are recommended.

The detailed protocols for membrane preparation and protein deglycosylation are given elsewhere [8]. Briefly, after washing with ice-cold PBS, ~ 80–95% confluent cells are scraped into PBS with 1 × protease inhibitors (μg/ml): 1 leupeptin; 2 aprotinin; 50 Pefabloc; 121 benzamidine; 3.5 E64. Cells are collected in 50-ml tubes by centrifugation at 1000 × *g* for 5 min at 4 °C. Cells are resuspended in lysis buffer (10 mM HEPES pH 7.2; 1 mM EDTA; 1 × protease inhibitors) and incubated on ice for 10 min. Cell lysis is performed with a pre-cooled Dounce cell homogenizer (8–10 strokes) and lysis is monitored with a microscope after trypan blue stain. An equal volume of sucrose buffer (10 mM HEPES pH 7.2; 500 mM sucrose; 1 × protease inhibitors) is added and the same number of strokes repeated, as above. Cells are centrifuged at 6000 × *g*, 10 min at 4 °C to pellet debris and organelles and the supernatant is centrifuged again at 100,000 × *g*, for 30 min at 4 °C to collect membranes.

For Endo H digestion, 20–100 μg membrane protein is solubilized in 50–100 μl Endo H incubation buffer [8] with

1 × protease inhibitors. Endo H (2 μl or 10 mU; Roche) is added to the reaction mixture and incubated 3 h to overnight at 37 °C.[1]

For PNGase F digestion, a solution of 20–100 μg protein in 50–100 μl PNGase F incubation buffer (20 mM sodium phosphate pH 7.5) with 1 × protease inhibitors is prepared. A 1/20 volume of denaturation solution (2% (w/v) SDS, 1 M β-mercaptoethanol) and of 15% (w/v) NP-40 are added) are added. The deglycosylation starts by the addition of 4 μl (10 mU) of PNGase F (GLYKO®/ProZyme; San Leandro, CA, USA) to the reaction mixture and samples are incubated 3 h to overnight at 37 °C.

If necessary the deglycosylated samples may be precipitated and concentrated by the addition of 4 volumes of cold ethanol (− 20 °C). The nonglycosylated, core-glycosylated and complex-glycosylated form of CFTR can be separated by SDS-PAGE and subsequently detected by WB (see above).

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