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Localization of the cystic fibrosis transmembrane conductance regulator in airway secretory glands

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ABSTRACT: Cystic fibrosis (CF) is caused by mutations in the gene coding for the CF transmembrane conductance regulator (CFTR). From human normal tracheal submucosal gland cells in culture, we identified endogenous CFTR as a 170 kDa protein, consistent with that of fully glycosylated, mature CFTR molecule. This observation led to the hypothesis that airway secretory glands could be an important site for the CFTR expression.

Using anti-human CFTR polyclonal and monoclonal antibodies, we examined the cellular and subcellular localization of the CFTR protein in airway submucosal glands from human and bovine tracheal tissues as well as in tracheal gland cell cultures.

In human tracheal tissue, CFTR immunolabelling was present along both the apical and basolateral plasma membranes of glandular mucous cells. In contrast, CFTR was associated with the secretory granules of glandular serous cells. Using immunogold electron microscopy, we demonstrated that CFTR protein was more specifically associated with the membrane of serous cell secretory granules. In bovine tracheal tissue CFTR labelling was also identified in the secretory granules of glandular serous cells. In contrast, when bovine and human tracheal gland cells were cultured, no mature secretory granules were present, but a predominantly intracytoplasmic distribution of CFTR was observed.

Our data thus suggest that in airway tissues, CFTR could be involved in intracellular processes of the mucus exocytosis in submucosal secretory glands.
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Cystic fibrosis (CF) is characterized by a defect in cyclic adenosine monophosphate (cAMP)-regulated chloride conductance [1, 2] and is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) [3–5]. In the human, analysis of CFTR at the messenger ribonucleic acid (mRNA) and protein level suggests that the protein is mainly expressed in epithelial tissues, such as the ducts of salivary glands and of the pancreas, as well as in the kidney tubules and intestinal crypts where Cl^- and fluid secretion occur [6–11]. Immunolocalization and cell fractionation studies of the CFTR protein in these tissues and in cultured epithelial cell lines (T84, HT29) and human airway immortalized surface epithelial cells have demonstrated that CFTR is a plasma membrane protein [12–14]. These observations, together with the functional analysis of recombinant CFTR expression [5] suggest that CFTR is an apical membrane chloride channel. In CF, a critical site for the clinical expression of the disease is the lung, where severe obstruction of the conductive airways occurs due to the presence of dehydrated and sticky mucus. Human airway surface epithelial and submucosal gland cells are the main contributors to the secretion of mucus and exhibit regulated chloride secretion [15, 16]. As a result, both surface and submucosal secretory cells may be implicated as potential sites of the primary airway defect in CF. Both Ca^{++} and cAMP dependent regulation of Cl^- secretion was shown to be defective in CF tracheobronchial gland cultures in comparison to normal gland cultures [17].

We have recently shown that the CFTR protein was restricted to the apical compartment of the ciliated cells in normal respiratory tissues whereas in homozygous ΔPhe 508 CF patients, CFTR markedly accumulated in the cytosol of the surface epithelial cells [18]. Because CFTR is involved in the control of Cl^- secretion in the human airway surface epithelial cells [15], it is of interest to determine whether the CFTR protein is also expressed in airway secretory glands.

Therefore, we addressed several questions: Do tracheal submucosal glands express CFTR protein? If so, is it the entire CFTR glycoprotein? Does its location differ between serous and mucous type gland cells? Does its expression in bovine tracheal submucosal glands resemble that in human tracheal glands? Do cultured human and bovine gland cells model the situation in native glands?

In the present study, polyclonal and monoclonal antibodies were used to demonstrate that in tracheal submucosal glands, CFTR was intracytoplasmic and more specifically associated with the membranes of the secretory granules of human and bovine glandular serous type cells. We also observed a cytosol immunolabelling pattern in cultured tracheal secretory gland cells.

Methods

Generation of anti-CFTR antibodies

A polyclonal antiserum directed against the R domain of CFTR (PATG-R) was raised upon immunization of rabbits with a specific *E. coli* fusion protein as previously described [18]. Two anti-CFTR monoclonal antibodies named MATG 1031 and MATG 1061, were raised against peptides corresponding to CFTR amino acids 107–117 and amino acids 503–515 (ΔPhe 508), respectively. In earlier studies, the specificity of the PATG-R and MATG preparations was confirmed by immunoprecipitation of *in vitro* expressed CFTR protein [18, 19] and by the immunocytochemical recognition of CFTR in cells expressing recombinant CFTR [18]. The specific recognition of the 140 kDa and 170 kDa forms of CFTR by MATG 1031 was demonstrated by Western blotting. Immunocytochemical detection of CFTR by means of the PATG-R and MATG 1061 on human airway surface epithelial cells gave a positive and specific immunoreactive signal [18].

Cell cultures

Normal human tracheal segments were obtained from young healthy adults who died from head trauma. Fresh bovine tracheae were obtained from a local abattoir. Isolation and culture conditions of bovine tracheal submucosal gland (BTG) [20] and human tracheal submucosal gland (HTG) [21] cells have previously been described.

SDS-PAGE and Western blotting

Whole cell protein extracts were prepared from cultured HTG and BTG cells as follows: cells were scraped into an ice-cold Tris buffer (50 mM TrisHCl; pH 7.5 containing 1.0 mM phenylmethysulphonylfluoride (PMSF), precipitated overnight at 4°C with trichloracetic acid (5% w/v final concentration) and centrifugated for 10 min at 10,000 g. The pellet was then dissolved in a SDS-PAGE disaggregation buffer (50 mM Tris, pH 6.8 with H_3PO_4, 2% SDS (w/v), 15% glycerol (w/v), 2% β-mercaptoethanol and 1 mM ethylene-diaminotetracetic acid (EDTA)), as recently recommended for CFTR solubilization and extraction of airway epithelial cells [22]. Samples were dissolved at room temperature at a protein concentration of 5 μg/μl. Proteins (100 μg/well) were separated by electrophoresis on SDS/5% polyacrylamide gels, transferred to nitrocellulose and then probed with a 1:100 dilution of MATG 1031. Western blots were developed by the enhanced chemiluminescence (ECL) method, using the ECL kit (Amersham). A high degree of similarity has already been shown in the amino-acid sequence between the human and bovine CFTR [23].

Light and confocal immunofluorescent microscopy

For immuofluorescence staining, BTG cells at passage 3 were grown on collagen IV-coated glass coverslip for 10 days. For the HTG cell culture, cells at passage 2 were seeded on 9.0 mm diameter, 0.45 μm pore size filter (Cyclopore membrane, Falcon) at a density of 5×10^5 cells·filter^{-1}. Confluent filter-grown HTG cells (day 10) showed characteristics of epithelial and secretory cells including cytoplasmic staining for cytokeratin and for two secretory protein markers of the glandular serous-cell type, *i.e.*, lysozyme and antileucoprotease [21]. All human and bovine tracheal samples were immediately frozen in liquid nitrogen after collection. For CFTR localization by light microscopy, frozen sections (5 μm) were air dried and rehydrated in phosphate buffer saline (PBS) 0.1M before the immunohistological studies. Cell cultures were fixed in methanol for 30 min at -20°C. The fixed tissue sections and cell cultures were then rinsed in PBS-bovine serum albumin (BSA) for 5 min and incubated at room temperature for 60 min with MATG 1061 at a dilution of 1:400 in PBS-BSA. After two washings in PBS, the sections were incubated for a further 45 min with an anti-mouse IgG biotinylated complex at a dilution 1:50 in PBS-BSA solution and then incubated for 30 min with streptavidin-FITC (Amersham, U.K.) at a dilution of 1:50. The samples were observed with a Zeiss Axiophot (Le Pecq, France) microscope using successive epifluorescence and Nomarski differential interference illumination. Appropriate controls were used in which the primary antibody was omitted or replaced by a non-immune mouse antiserum. For confocal microscopy, a series of optical sections of tracheal submucosal glands labelled with PATG-R and MATG 1061 conjugated with an fluorescein isothiocyante (FITC) second antibody were imaged by a BIORAD MRC 600 (Ivry/Seine, France) confocal laser scanning microscope using an Argon Ion Laser (488 nm). All images were collected with a Zeiss X63, 1.4 NA, oil objective. Sequential serial sections were collected as XZ sections at 1.2 μm

intervals. This means that the sections were parallel to the apical/basolateral axis. In order to enhance the visibility of the labelling localization, a pseudo-red colour was given to the FITC labelling using a grey scale ramp (0–255). The red colour corresponded to the values ranging from 37 to 255.

Transmission electron microscopy

Human and bovine tracheal tissue samples were fixed in 4% paraformaldehyde in PBS. Samples were then embedded in Lowicryl K_4M or in epoxy resin. Ultra-thin sections (0.07 µm thick) were cut on a Reichert ultramicrotome (Ultracut E, Leica, Rueil-Malmaison, France) and mounted on gold grids for further immunogold labelling. In order to reduce the nonspecific background, we used the technique developed by DURRENBERGER *et al* [24]. The coupling of IgG to 15 nm protein A-gold solution was carried out immediately before CFTR labelling. Sections were incubated for 10 min in an ovalbumin solution (2.5% in PBS 0.1M, pH 7.2) and incubated for 1 h with freshly sonicated pre-coupled IgG protein A-gold solution. The grids were then stained with uranyl acetate and lead citrate and observed using an Hitachi H 300 electron microscope (Elexience, Verrières Le Buisson, France) at 75 kV. In order to identify the glandular cell types (mucous or serous cell) involved in the CFTR expression more precisely, lysozyme, a specific serous cell protein marker, was identified using the biotin-streptavidin-gold technique with a lysozyme antiserum. The grids previously labelled with the 15 nm gold-protein A complex were floated on a droplet of 12% (w/v) ovalbumin (Sigma; St Louis, MO) in 0.01 M PBS, pH 7.2, for 5 min, and transferred for 1 h at room temperature onto drops of rabbit anti-human lysozyme IgG antibody (Boehring, Somerville, NJ; dilution 1:50). The grids were rinsed for 10 min in PBS 0.01 M, pH 7.2, and for 5 min in PBS/1% ovalbumin, pH 7.2. The grids were then transferred onto drops of a 250-fold dilution of biotinylated goat anti-rabbit IgG (Janssen; Beerse, Belgium) in 1% ovalbumin/PBS for 1 h and floated again onto PBS. The sections were floated onto a drop of a five-fold dilution of 5 nm colloïdal gold-streptavidin (EY Labs; San Mateo, CA) in 1% ovalbumin/PBS for 1 h. The grids were rinsed in PBS and deionized water and then dried. In order to assess the specificity of lysozyme labelling, controls were performed by omitting the biotinylated goat anti-rabbit IgG incubation.

Analysis of the spatial distribution of CFTR and lysozyme labelling

The specific localization and spatial distribution of CFTR and lysozyme were analyzed using 15 nm (CFTR) and 5 nm (lysozyme) gold particle markers. Images of labelled secretory cells obtained by electron microscopy were digitized with a charge compelled device (CCD)-camera fitted to an image analyzer (Bio500, BIOCOM, Les Ulis, France). Then, a software specifically developed for quantitative cytochemistry [25] was used as follows. Firstly, the gold particles were isolated from the image background using two different procedures: a simple grey-level thresholding for 15 nm particles with a very high contrast and a top-hat procedure for 5 nm particles with a weaker contrast. Secondly, the outline of the secretory granules was drawn by hand. Finally, the distance from each pixel to the nearest edge was automatically computed. From this distance value, 5 concentric zones were automatically defined inside the granules and the number of gold particles (5 nm as well as 15 nm) falling inside each of these concentric zones were automatically counted. An illustration of this procedure is shown in Figure 1. By repeating this procedure in several secretory granules, we were able to quantify the relative percentage of each marker inside the 5 concentric zones.

Fig. 1. – Distribution of CFTR (15 nm) and lysozyme (5 nm) gold particle markers within 5 concentric zones of a serous cell secretory granule. The markers were extracted from a digitized image. The contours were automatically computed from the distance of each pixel to the edge of the granule.

Results

Western blotting analysis

To assess whether CFTR was present in tracheal secretory glands, MATG 1031 was used in an immunoblot analysis to detect cross-reactive proteins in cultured HTG cell lysates. As shown in Figure 2, lane A, an immunodetectable 170 kDa protein band

was present in detergent-solubilized HTG cell lysates; no 145 kDa protein band was identified. Several small polypeptides were also detected which one could presume to be the result of either CFTR degradation products or alternative splicing [10, 26]. Several arguments indicate that the 170 kDa protein band is CFTR. First, fibroblasts expressing the recombinant normal CFTR [19, 27] (Fig. 2, lane B) or ΔPhe 508-CFTR (Fig. 2, lane C) showed a positive immunolabelling with MATG 1031, whereas control cells lacked this labelling (Fig. 2, lane D). The predominant recombinant CFTR form was recognized as a 145 kDa protein, the non-glycosylated CFTR in cells expressing recombinant CFTR [19, 28]. The fully glycosylated mature CFTR, which has an apparent mobility of 170 kDa was also recognized by MATG 1031 and corresponded to the 170 kDa band observed in HTG cells (Fig. 2, lanes A, B). Secondly, the 165–170 kDa protein band was observed both in BTG cell lysates and in cultured respiratory surface epithelial cell lysates from human normal nasal polyps (data not shown). Thirdly, recent findings support the conclusion that the endogenous, mature CFTR in human airway epithelium from adult tracheal and nasal immortalized surface epithelial cells has an apparent molecular mass of about 165–185 kDa as determined by Western blotting analysis [13, 22].

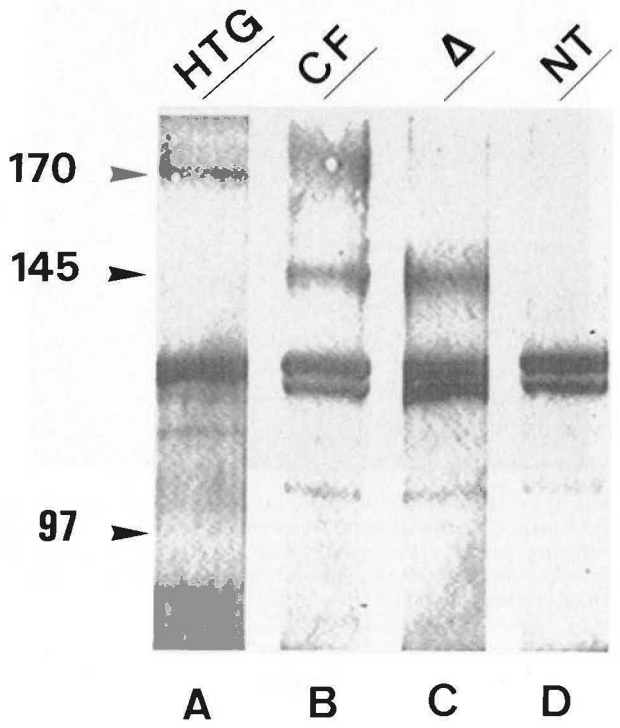

Fig. 2. – Western blot analysis of CFTR and ΔPhe 508 CFTR with a monoclonal antibody (MATG 1031). Lane A: Total protein extracts of cultured human tracheal gland cells (HTG). Lanes B, C and D: Total protein extracts of fibroblast cells expressing normal recombinant CFTR (CF) or ΔPhe-CFTR (Δ) as well as non-transfected control cells (NT). The 145 kDa and 170 kDa protein bands correspond to nonglycosylated (the predominant form in cells expressing recombinant CFTR) and fully glycosylated CFTR forms, respectively.

Localization of CFTR by light and confocal microscopy

Cryofixed preparations of human tracheal submucosal tissue showed that secretory glands were mainly composed of mucous cells, whereas in bovine tracheal glands, the serous-cell type was the most representative (Fig. 3B and D, respectively). In human tracheal glands (Fig. 3A), MATG 1061 labelling showed that the CFTR protein was present along the apical and basolateral plasma membrane of human gland mucous cells. We also observed a labelling in cells which had the appearance of serous-type cells but we could not assert whether the CFTR was localized on secretory granules and/or intracytoplasmic organelles. In bovine tracheal tissue (Fig. 3C), CFTR was identified on secretory granules of glandular serous-type cells. In order to examine the 3-dimensional distribution of CFTR in tracheal glands, we used a confocal laser scanning microscopy which allowed us to visualize CFTR labelling throughout the gland as a series of consecutive intracellular sections [29]. The twelve serial confocal sections through the bovine tracheal gland cells (Fig. 3E) showed that most of the fluorescence (with pseudo-red colour) was identified throughout the secretory cells with no preferential localization in the luminal apical cell membrane lining the duct. Nevertheless, it was impossible by either light or confocal laser scanning microscopy to precisely define with which intracellular structure the CFTR was associated. In cultured BTG cells (Fig. 3F) no secretory granules were present, but a predominantly intracytoplasmic distribution of CFTR was also observed after immunolabelling staining. A diffuse intracytoplasmic staining pattern was observed when the PATG-R antibody was used on cultured HTG and BTG cells (data not shown).

Localization of CFTR by electron microscopy

As shown in Figure 4A and B, immunogold labelling with MATG 1061 confirms the subcellular localization of CFTR in human and bovine tracheal secretory gland cells with 15 nm gold particles preferentially observed in the vicinity of the plasma membrane of mucous cells and serous secretory granules. In addition, double labelling of CFTR and lysozyme, with 15 nm and 5 nm gold particles respectively, allowed us to define the human secretory cell type (serous or mucous) and to identify the distribution pattern of CFTR in these secretory cells. The CFTR labelling was mainly present at the periphery of the human lysozyme-positive (serous cell) secretory granules whereas lysozyme labelling was homogeneously distributed inside the human serous secretory granules (Fig. 4A). In the mucous secretory cells, lysozyme was not identified and some labelling of CFTR was present along the plasma membrane surrounding the cell (data not shown).

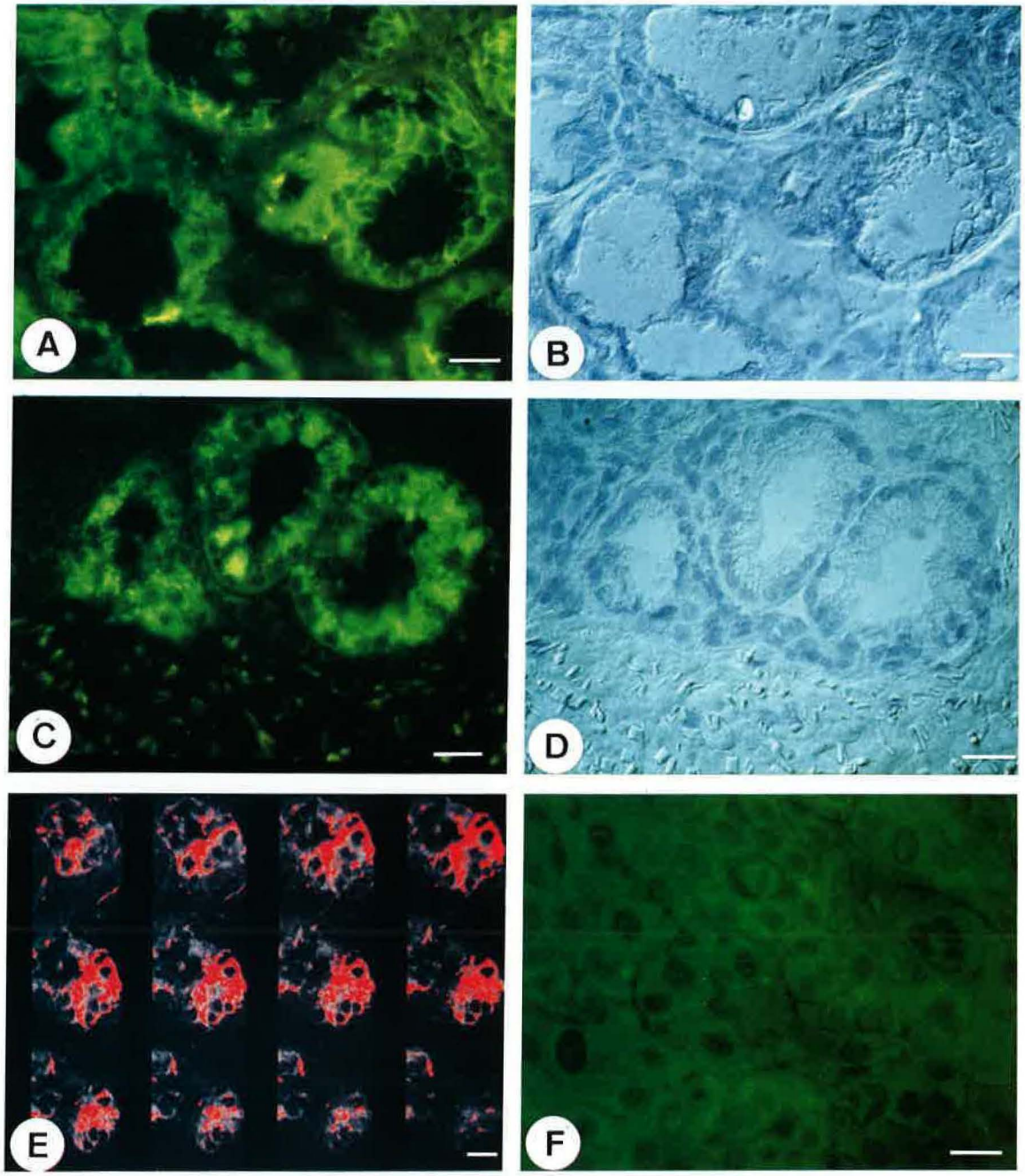

Fig. 3. – Immunofluorescent and Nomarski interference optic photomicrographs of normal human and bovine tracheal gland cells. Immunolabelling of CFTR of human (A) and bovine (C) gland cells as well as in cultured bovine tracheal gland cells (F) was performed with MATG 1061. Nomarski photomicrographs show the respective human tracheal gland mucous and serous cells (B) and bovine tracheal gland serous cells (D). Twelve serial confocal XZ sections through bovine tracheal gland cells (E). Bar: A, B, C, D = 100 µm, E = 10 µm, F = 200 µm.

Figure 1 represents an example of the distribution of 15 nm (CFTR) and 5 nm (lysozyme) gold particles inside a serous cell secretory granule. Each marker is displayed together with the contours of the 5 concentric zones (defined from a distance function), within the secretory granules. As reported in Table 1, the CFTR labelling (15 nm gold particles) was mainly concentrated in the outer zone of the secretory granules (72% of the CFTR labelling was identified in the 2 outer zones). This suggests that there is an association between CFTR and the membrane of serous cell secretory granules. On the other hand, the lysozyme labelling (5 nm gold particles) was uniformly distributed within the 5 concentric zones without any preferential distribution.

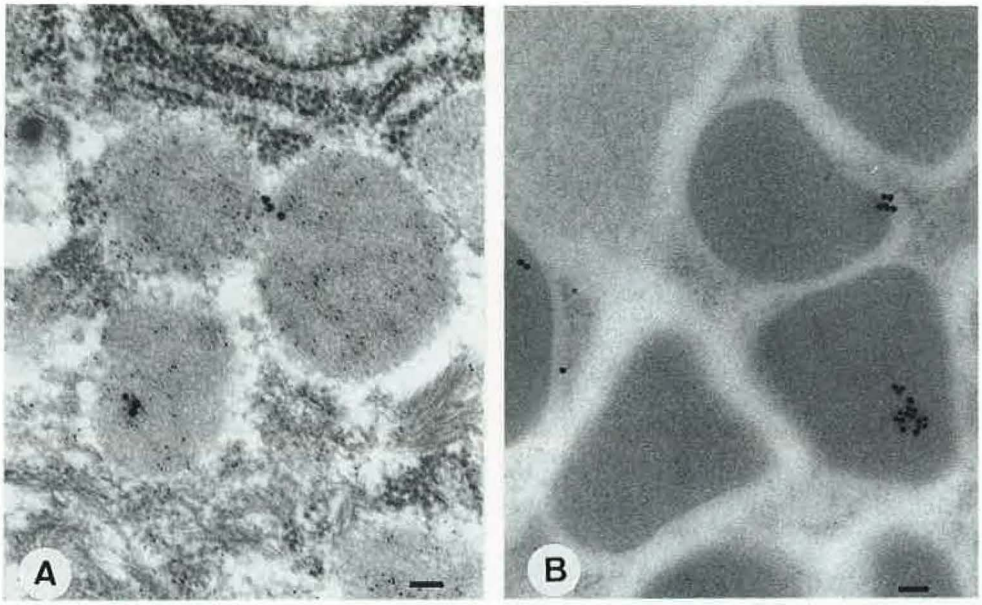

Fig. 4. – Transmission electron micrographs of normal human (A) and bovine (B) tracheal gland serous cells. (A) Human serous cell sections were initially immunolabelled for CFTR with 15 nm protein A-gold particles and then subsequently for lysozyme with 5 nm protein A-gold particles. (B) Bovine serous cell sections were immunolabelled for CFTR with MATG 1061. A strong labelling for lysozyme was present throughout the secretory granules (A). Note that the CFTR labelling is only detected along the periphery of the secretory granules (A, B). Bar = 0.1 μm.

Table 1. – Spatial distribution of gold particles inside serous-type secretory granules

	Spatial distribution in zones 1–5 %				
	1	2	3	4	5
CFTR	46	26	14	14	0
Lysozyme	13	22	29	22	14

Percentage of gold particles for the CFTR (15 nm) and lysozyme (5 nm) labelling within 5 concentric zones (1→5: outer→centre) inside serous-type secretory granules. The spatial distribution of the CFTR and lysozyme gold particle markers in the 5 concentric zones was calculated from 13 (double labelling of CFTR and lysozyme) and 6 (single CFTR labelling) secretory granules. CFTR: cystic fibrosis transmembrane conductance regulator.

Discussion

Using anti-human CFTR polyclonal and monoclonal antibodies, we have investigated the subcellular and cellular distribution of the CFTR protein in normal airway submucosal tissues. In the human, the CFTR protein is shown to be present along the apical and basolateral plasma membranes of the mucous secretory gland cells; in contrast, in the glandular serous cells, CFTR is localized to the membrane of secretory granules. The localization pattern is similar, using two antibodies raised against different domains of the CFTR protein, and suggests that the observed labelling accurately reflects the distribution of CFTR in airway secretory glands. Interestingly, the basolateral plasma membrane and intracytoplasmic distribution of CFTR in tracheal gland secretory cells obtained with PATG-R and MATG 1061 antibodies differs on normal human nasal and tracheal surface airway epithelium where the signal is restricted to the apical plasma membrane domain of ciliated cells [18]. Recently, KARTNER *et al.* [30] have also demonstrated that the CFTR protein is confined to the apical membrane of normal human sweat gland cells. Since serous and mucous cells are difficult to distinguish by light microscopy, we have used a specific serous cell marker (the lysozyme) which allowed us to identify the serous cell type by the presence of lysozyme-positive secretory granules. By using immunogold electron microscopy and a computer analysis of the spatial distribution of CFTR and lysozyme labelling, we have shown that CFTR labelling is more specifically located in secretory granule associated-membranes of the glandular serous cells in human and in bovine airway glands. By Western blotting analysis, a monoclonal antibody directed against the CFTR protein (MATG 1031) identifies a protein band from cultured HTG cells with an apparent molecular mass of about 170 kDa, consistent with that of fully glycosylated, mature CFTR [19, 28]. In contrast to the CFTR labelling in secretory granules of the glandular serous cells, CFTR labelling in cultured HTG and BTG cells is mainly intracytoplasmic. These results support the hypothesis that, in our culture conditions where no mature secretory granules were present in cells, the localization pattern of CFTR is different from that observed in well differentiated glandular serous cells.

These results demonstrate that airway submucosal glands represent another potentially important site in

which CFTR can function. Our findings favour the hypothesis that, in normal airway tissues and more specifically in respiratory gland cells, CFTR has additional possible roles. The involvement of the CFTR protein in intracellular protein trafficking has been suggested by BARASCH *et al.* [31]. The latter authors also suggest that the defective acidification of intracellular organelles, observed in immortalized CF nasal polyp cells, is potentially caused by a defective vesicular Cl^- channel. This can, in turn, be responsible for the abnormalities in sialylation and sulphation of mucous glycoproteins, previously observed in CF patients. According to VERDUGO [32], the release of respiratory mucus is mainly regulated by the exchange of ions and water across the membrane of the secretory granules, based on the Donnan swelling mechanism. During the process of exocytosis, the increase of intragranular water, required for mucin swelling and for the formation of the hydrated gel phase, may be controlled by ion and water exchange regulated by the secretory-granule membrane. Thus, a defect in CFTR function in secretory granules of airway gland cells could lead to an extremely slow rate of swelling with a resultant mucus gel that remains thick and therefore difficult to discharge into the airway lumen.

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