Epithelial IgG and its relationship to the loss of F508 in the common mutant form of the cystic fibrosis transmembrane conductance regulator

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The most debilitating feature of cystic fibrosis (CF) disease is uncontrolled inflammation of respiratory epithelium. The relationship between the commonest mutated form of CFTR (F508del or AF508) and inflammation has not yet been elucidated. Here, we present a new paradigm suggesting that CFTR can interact with intra-epithelial IgG, establishing a direct link between normal CFTR and the immune system. Further, our data show that the amino-acid sequence local to F508 can bind IgG with high affinity, dependent on F508, such that loss of F508 abolishes this link both in vitro and in the intact cell.

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1. Introduction

Most cystic fibrosis (CF) arises from a single amino-acid deletion (AF508) in the cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7). The resulting multi-system disease has many unexplained features including disordered innate immunity [1,2]. F508 is in the first nucleotide-binding domain (NBD1) of CFTR but no significant structural alterations correlate with its deletion [3]. Since the F508 region forms an accessible loop, we speculated that this region might participate in protein–protein interactions. We therefore looked for candidate proteins in tracheal epithelium from AF508CFTR or CFTR-null transgenic mice [4]. During our control immunohistochemical studies of tracheae, we noticed differential staining in the ‘non-specific’ background when secondary anti-IgG antibodies were applied in the absence of primary. This differential distribution segregated as expected for CFTR itself [5], suggesting that a CFTR-induced antibody-binding site was present. CF patients are mucosally infected with a restricted set of pathogens (see discussion) and some authors believe CF epithelia are spontaneously inflamed in the absence of infection [6]. CF cells secrete IL-8 in excess over wild-type due to activation of NFkB [7] and IgG levels are abnormally elevated in the CF gut lumen [8]. The serendipitous data presented here suggest that IgG binds to the F508 region of CFTR, F508-dependently.

2. Materials and methods

2.1. Immunohistochemistry

Wax-embedded tracheal sections from wild-type, CFTR-null or homozygous AF508 CF mice were mounted on glass slides and stained using dianamobenzidine [9]. HRP-labelled Protein-G (1 μg/ml; Sigma P8170) was used to stain tissue for endogenous IgG.

2.2. Dotblots

Peptides (10 nmol/dot) (KENIFGVSYDEYRYS (wild-type CFTR peptide 503–519), KENIFGVSYDEYRYS (AF508 CFTR peptide 503–518); SZ, GQNHKTTASTRK (CFTR 780–793); KE, KEHYVDLKDRPPFA (NDPK-A 49–62); GE, GETNPADKPGTIR (NDPK-A 92–105)) were immobilised on nitrocellulose membranes using a Whatman-Biometra Hybrislot suction manifold. Membranes were blocked using 1% BSA then incubated with 1:1000 dilutions of HRP-labelled secondary antibodies (Sigma). After

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washing in PBS with 0.1% Tween 20, bound antibody was visualised using enhanced chemiluminescence. \(^{125}\)I-labelled purified mouse monoclonal IgG was produced by the chloramine-T method [10]. This IgG was repurified by gel filtration; only a single iodinated 150 kDa band was present after autoradiography/non-reducing SDS–PAGE.

2.3. BIAcore

Peptides were amine-coupled to a CM5 chip using standard methods [11]. The four available channels were coupled with a 10 \(\mu\)M solution of either KENIFGVSYDEYRYS (wild-type CFTR 503–519, KENIGCVSYDEYRYS (ΔF508 CFTR 503–518), GTFIKENIWKGRTYTRII (Varicella Zoster Virus E1B 326–344), QRVGLGRTSGKSTLL (control NBD2 CFTR 1237–1253) or the final channel was left blank as a baseline control. Analyte was passed over each channel in this order; the signal from the final channel (NBD2 or blank) was automatically subtracted from all other traces. IgG (10–40 \(\mu\)M; Sigma I4506) was used as analyte in proprietary Biacore buffer. Biacore software was used to calculate \(K_D\); \(\chi^2\) values were within prescribed confidence limits.

2.4. Immunofluorescence

Nasal cells from patients undergoing unrelated surgery (local ethical committee approved) were fixed in 4% paraformaldehyde, incubated in 1 mM glycine for 15 min, permeabilised using 1% Triton X-100, washed three times in PBS, then blocked in 5% donkey serum for 15 min. Cells were incubated in primary antibodies (FITC-labelled anti-hIgG (Jackson) and mouse anti-CFTR NBD1 (Neomarkers, Ab-3) at 1:100) at room temperature, with shaking, overnight. After three washes in PBS, cells were stained with rhodamine-labelled anti-mouse IgG (Jackson, 1:100). After 2 h incubation, with shaking, the cells were washed five times in PBS and mounted (6% n-propyl gallate, 70% glycerol, 100 mM Tris/HCl, pH 7.4). Coverslips were sealed with nail varnish for image capture using a Zeiss 510 laser-scanning confocal microscope.

2.5. Precipitations

Ovine tracheal epithelial membranes were prepared and detergent-extracted as previously described [12]. A centrifuged extract was used for precipitation with protein-G Sepharose beads (Pharmacia), i.e. the sample generated was the equivalent of the normally-discarded ‘pre-clearing’ step applied in immunoprecipitation protocols to eliminate non-specific, protein-G interactions. Here we required these interactions to be present and used competitive CFTR-peptide incubations to provide specificity. Twenty micrograms of detergent-extracted protein was incubated with 5 \(\mu\)l of protein-G (or A) Sepharose bead slurry ± peptide, and vortexed overnight at 4 °C. The precipitates were washed and phosphorylated with PKA and [\(^{\gamma}\)P]-ATP to visualise CFTR protein [13]. One unit of PKA catalytic subunit (Calbiochem–Novabiochem) was incubated with each sample in 50 \(\mu\)l of 50 mM Tris–HCl (pH 7.5), 10 mM MgCl\(_2\), 100 \(\mu\)M unlabelled ATP, 10 \(\mu\)Ci [\(^{\gamma}\)P]-ATP (30 °C, 30 min) with vortexing. The phosphorylated beads were subjected to SDS–PAGE and the \(^{\gamma}\)P-labelled bands visualised using a Packard InstantImager.

3. Results

Tracheal staining using peroxidase-labelled protein G intensely stained the apical membrane of wild-type mouse tracheal epithelium (Fig. 1A, brown colour wild-type left panel, representative of \(n = 3\)). Attenuated or absent membrane staining was observed at the apical membrane of two different CFTR mutant tissues (CFTR-null or ΔF508-CFTR, respectively). Aggregations of protein G staining were observed in the null tissue (red arrows). Since the sole difference between these epithelia was the absence (CFTR-null) or mutation (ΔF508) of CFTR, these data suggest not only that murine IgG is localised to the apical membrane in wild-type trachea, but also that CFTR mutation can affect expression and localisation of epibetal IlgG. We confirmed this differential staining using secondary anti-IgG antibodies alone (not shown).

To localise the defect, peptides were synthesised from human CFTR local to F508, amino acids 503–516 in CFTR. These and control peptides from other parts of CFTR or unrelated proteins were each immobilised on nitrocellulose and overlaid with different IgG preparations. Fig. 1B shows binding of purified IgG to immobilised wild-type (KF) peptide (referred to as KENIIF, where F is F508) compared to the equivalent ΔF508 (KI) sequence (referred to as KENII) and three control peptides. We found that three unrelated peroxidase-labelled anti-IgG antibodies recognising different species all bound specifically to KENII, minimally to KENIF and did not bind control peptides (Fig. 1B, top three panels). To exclude the possibility that the interaction was with peroxidase, a pure mouse monoclonal \(^{125}\)I-labelled IgG was overlaid and found to bind wild-type KENII peptide (Fig. 1B, bottom panel). Thus, in vitro, peptides corresponding to the F508 region of CFTR can bind IgG provided F508 is present.

To quantify this in vitro interaction we applied human IgG to the BIAcore system, which utilises surface plasmon resonance to validate protein–protein interactions in real time. We immobilised KENII, KENII or a control peptide on Biacore chips and perfused human IgG at two different concentrations as analyte. \(K_D\) was determined to be 69 nM for the KENII peptide and 86 nM for the KENII peptide (Fig. 2A). Similarity searches revealed significant homology between the KENII region of CFTR and antibody-binding domains of protein L, a bacterial virulence-related protein [14,15].
Protein L interacts specifically with κ light chains, whereas the more commonly used antibody-binding proteins (A/G) both bind the heavy chain of IgG.

We compared the binding of KENII, KENII and protein L to κ- and λ-containing isoforms of IgG (Fig. 2B). Identical amounts of total human IgG or three isoforms of IgG were immobilised on 96-well plates and screened for biotinylated peptide binding.

**Fig. 2.** (A) Top, BIAcore traces for binding of human IgG (40 nM, analyte) to immobilised peptides. (A) Bottom, BIAcore traces for binding of human IgG (10 nM, analyte) to immobilised peptides. (B) Data comparing binding of biotinylated KENII, KENII and protein L to immobilised isoforms of IgG and IgA.

**Fig. 3.** (A) Immunofluorescent staining for human IgG (FITC) and CFTR (rhodamine) using either wild-type (left panels) or homozygous ΔF508 primary ciliated nasal epithelial cells (right panels). Far right panel; no primary antibody control (wild-type). Scale bar, 5 μM. Images are representative of n = 4 experiments. (B) As in A but wild-type cells previously incubated with myristoylated (myr) KENII or KENII peptide. Images are representative of results from three different experiments.
Biotinylated KENIIF peptide bound them all with equal efficacy, KENII showed a significantly reduced interaction, whereas IgA bound KENIIF, KENII and protein L with equal efficacy. Protein L bound IgG containing the \( \kappa \) (but not \( \lambda \)) light chain as expected. These data confirm that KENIIF peptide can bind IgG and that F508 regulates binding. Further work will have to establish the number of sites where this interaction takes place but judging by our recent experience with the complex interaction of the multimeric protein kinase CK2 with the very same KENIIF region of CFTR, this is likely to involve more than just one motif on the interacting protein \[16\]. Our working hypothesis is that this region can adopt more than one conformation.

Next, we investigated whether IgG localises with CFTR in vivo using immunofluorescence and laser-scanning confocal microscopy. We stained wild-type human ciliated airway cell biopsies \[17\] with anti-human IgG (FITC-label) and anti-human CFTR (rhodamine-label). Fig. 3A shows that CFTR and IgG staining colocalises specifically at the ciliated apical membrane of wild-type cells. Staining for both proteins was absent in cells from three \( \Delta F 508/\Delta F 508 \) CF patients. To determine whether the F508 region is responsible for the observed colocalisation, we attempted competition with cell-permeant KENIF/KENII CFTR peptides. KENIIF and KENII peptides were myristoylated (myr-) and the ciliated cells were incubated in the presence of 10 \( \mu M \) peptide for 4 h prior to fixation and staining. Incubation with myr-KENIIF (but not myr-KENII) peptide abolished the colocalisation (Fig. 3B).

We theorised that, if CFTR is bound to IgG in vivo, protein-G Sepharose should coprecipitate IgG and CFTR from epithelial membrane extracts without needing an antibody to CFTR. Protein-G precipitation from sheep tracheal membrane extract \[18\] pulled down a PKA-phosphorylatable band at the molecular weight of CFTR that was not present in an identical protein-A precipitate (Fig. 4A). Since protein G binds ovine IgG, but protein A does not bind as efficiently, the simplest interpretation was that the phosphorylatable band interacted with an endogenous, ovine, membrane-associated IgG. Again, the protein G interaction was inhibitable with KENIIF, but not KENII or another peptide, specifying the interaction to this region (Fig. 4A). These experiments also confirmed that the interaction is with endogenous antibody and not an artefact of the tagged antibodies. Thus, across mouse, sheep and human airway epithelia, the data consistently suggest that CFTR can bind IgG. The similarity between CFTR and protein L suggests that these IgG-binding molecules may have a similar mode of interaction.

Protein L is not the only molecule having sequence homology with this KENIIF region; Fig. 5 shows that certain pathogenic bacterial proteins also contain related sequences; we noticed a striking similarity in a poxviral protein (I6, below, and Fig. 6).
We synthesised the KENIIF-equivalent peptide from poxvirus (Variola I6, P33002 326–342) and tested it for interaction with IgG using BIAcore. Fig. 4B shows that the IgG interaction with Variola I6 peptide was the strongest we had tested.

4. Discussion

Our studies have produced unexpected results that relate to the pathway between ΔF508 and the disordered immunity responsible for most CF morbidity. The combined data point to an antibody-binding site in CFTR and suggest that CFTR’s F508 status may affect apical localisation of IgG. Our data demonstrate, by several independent methods, an F508-dependent interaction between CFTR and IgG. We have shown that the interaction is direct using BIAcore, and can occur in vivo because IgG both colocalises and coprecipitates with CFTR. Furthermore, the binding can be disrupted by exogenous KENIIF (but not KENII) peptide, strengthening the conclusion that this pathogenic region of CFTR binds IgG.

Apically-localised IgG provides a first line of defence against respiratory infections and our data suggest that this immune molecule is missing in most CF epithelia. Protein G, which binds IgG, is made by staphylococci to evade the immune response and nearly all CF babies develop Staphylococcus infection immediately after birth for unknown reasons. Mucosal inflammation may be hyperactive in CF to compensate for the missing IgG defence. Such a hyperactive compensation might also be present in heterozygotes giving them an advantage in resistance to epithelial infections. Interestingly, Fig. 5 shows that many infectious organisms contain similar sequences; one has been identified as a virulence factor. A protein kinase (CK2) known to regulate viral life cycles also interacts with NBD1 in an F508-dependent manner [16] and it may be that a complex interaction with multiple regulators of immunity occurs at this site which might relate to the locally restricted types of infections found in CF epithelia.

We speculate that the presence of an avid IgG-binding peptide in a virulent pathogen like smallpox suggests that it may have evolved this generic IgG-binding capacity either in order to sequester the epithelial immune mechanism or to harness it in some way to its own advantage. We were led to this speculation by epidemiological studies evaluating HIV-resistance genes (CCR5-D32) which are reported to be selectively amplified by smallpox outbreaks on a similar timescale and population frequency to ΔF508 [19]. These authors write: ‘Frequency of [CCR5-D32] is estimated ... in European populations. The allele is virtually absent in African, Asian, Middle Eastern, and American Indian populations... The geographic distribution... also implicates smallpox as the most likely historical source of selection... Scandinavian populations... were hit particularly hard by intense smallpox epidemics. Frequency of the [CCR5-D32] variant forms a north-to-south decline... with greatest prevalence in Scandinavian countries’. The frequency of the ΔF508 mutation follows an almost identical pattern and thus could be due to the same selective pressures [20]. This raises the possibility that the prevalence of CF is due to the greater survival of persons heterozygous for ΔF508 during smallpox epidemics, wherein childhood mortality reached 80%. Although the mechanism is unclear, we note that I6 is important in viral replication and viral capsid filling with viral DNA [21]; our observation that I6 can bind IgG additionally relates I6 to the host immune response, possibly using a similar mechanism to the organisms listed in Fig 5. Further work is needed to establish exactly how this mechanism functions.

We do not yet know which sites within IgG bind to the peptides reported here but it is becoming clear that this region of CFTR is on the surface of the nucleotide-binding domain where it is accessible.
to a number of partners. That F508 appears to be important for this interaction is interesting from a CF perspective. We suggest caution in interpretation of results from CFTR experiments that use antibodies, particularly from native epithelial tissues. For example, pre-clearing immunoprecipitations using protein G alone might discard a population of CFTR molecules of biological importance. Our data also add a potential caveat to trafficking therapies for DF508-CFTR. Such treatments may not restore IgG binding. Two decades after the discovery of CFTR, the mechanism responsible for CF disease remains disputed with some stating that ‘CF is an ion channel disease’ and others claiming that ‘CF is a disorder of immunity involving a restricted set of pathogens’. Our contribution is to highlight that the CFTR peptide sequence straddling the site of the commonest (by far) mutation around F508 bears striking similarities with a range of pathogen proteins. We hope that our studies into the diverse in vitro [16] and more recent in vivo [22] functions manifested by such peptides will fuel the debate on CF pathogenesis. Currently, there are three recognised ‘i’ factors in CF: ions, inflammation and immunity to which we now add a fourth, immunoglobulin. The latter is missing in most CF and we now need to determine the consequences for the patient.

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