CURRENT TOPIC

 Δ F508 in cystic fibrosis: willing but not able

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The membrane protein, cystic fibrosis transmembrane conductance regulator (CFTR), functions as an ion channel.^{1,2} It is located primarily in the apical plasma membrane of epithelial cells, where it acts as a 'gateway' for chloride ions to leave the cell after a rise in intracellular cAMP. In addition CFTR has a regulatory role over other ion channels in the cell membrane.^{3,4} CFTR is the product of a gene on the long arm of chromosome seven, the *CFTR* gene.

Cystic fibrosis is an autosomal recessive condition, caused by mutation of both *CFTR* gene alleles. Over 600 mutations of the *CFTR* gene have been identified, however one mutation, Δ F508, accounts for the vast majority of cystic fibrosis. Why does this unusual mutation, a codon deletion, have such high prevalence? Greater understanding of the molecular consequences of Δ F508 may answer this question and, more importantly, lead to therapeutic opportunities. This article will review the molecular biology of the Δ F508 mutation with particular reference to clinical implications.

Identification of the *CFTR* gene

Identification of the 'cystic fibrosis gene' was achieved by examining 'informative families' (with two or more affected children) for genetic linkage with a large number of genomic probes (short sequences of DNA that match one area of the human genome). This strategy relied on two assumptions; that adjacent genes tend to be inherited together through generations and that one mutation would predominate in this condition. In 1985 linkage of the 'cystic fibrosis gene' to markers on chromosome seven was reported.^{5,6} Calculations from the inheritance patterns indicated that two of the markers were relatively close and on either side of the gene. However there was still a lot of chromosomal ground to cover. It was four years until Tsui and his colleagues, using a technique that combined the mundane with the extraordinary (walking and jumping along the chromosome⁷), were able to publish the sequence of the gene.⁸

CFTR PROTEIN

The *CFTR* gene codes for a protein which contains 1480 amino acids and is a membrane ion channel. From the amino acid sequence a theoretical model of the molecular structure was developed. The molecule has several

Figure 1 (A) Different function domains of the CFTR protein. (B) Proposed structure of CFTR in the cell membrane (NBF=nucleotide binding fold).

distinct regions, the majority of which span the cell membrane (see fig 1). On the inside of the cell, connected to the membrane spanning domains, are two tightly folded regions that bind nucleotides. Between the nucleotide binding folds is situated a larger 'R' domain, a region with multiple sites for phosphorylation. The R domain and the nucleotide binding folds regulate chloride conductance through the channel. Over 600 mutations of the *CFTR* gene, that cause cystic fibrosis, have now been reported, but by far the commonest is a codon deletion that results in the loss of a phenylalanine residue at position 508 in the first nucleotide binding fold, the Δ F508 mutation.

The Δ F508 mutation

Approximately 50% of individuals with cystic fibrosis are homozygous for the Δ F508 mutation and in many countries, including Britain, over 90% carry at least one allele with the mutation.^{9,10} The prevalence of the mutation varies geographically with a white bias⁹ and is less common in other ethnic populations.¹¹ A number of DNA sequences, both within and adjacent to the *CFTR* gene, show remarkable

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conservation supporting the hypothesis that ΔF508 originated from a single mutational event.¹² Analysis of European genotypes suggests that this event occurred over 52 000 years ago,¹³ being introduced to Europe in the Palaeolithic period. Other population influxes in the Neolithic period may account for the heterogeneous European spread of the ΔF508 mutation.¹³

WHY IS ΔF508 SO SUCCESSFUL ?

ΔF508 is the commonest 'disease causing' mutation affecting white people. It seems likely that being a ΔF508 heterozygote offers biological advantage, as other causes of a high gene frequency would have been negated by the passage of time.^{13 14}

Assuming heterozygotes express approximately 50% of the normal amount of CFTR protein, their advantage may stem from reduced secretion of chloride (and water) when the intestine is challenged by cholera toxin, resulting in a reduced mortality during cholera epidemics.¹⁵ Two groups have investigated this theory on transgenic cystic fibrosis mice, heterozygous for a null mutation (that is one allele is unable to produce any CFTR). One group described a reduction in jejunal fluid accumulation after cholera toxin exposure in heterozygotes,¹⁶ whereas the other group,¹⁷ examining the effect of a variety of secretagogues (including cholera toxin) on ileal and colonic ion transport, were unable to detect any significant difference. If this hypothesis does account for heterozygote advantage it does not explain why ΔF508 should predominate over the other CFTR mutations. Experiments on transgenic mice carrying a ΔF508 allele may give a clearer answer.

A different explanation for ΔF508 advantage was suggested by the study of relatives of ΔF508 patients in the USA.¹⁸ Non-carriers were calculated to be three times more likely to develop asthma than ΔF508 heterozygotes. In a smaller cohort, heterozygotes for a non-ΔF508 mutation were eight times more likely to develop asthma. This protection from asthma was not found in a UK study of heterozygotes, identified through an antenatal screening programme.¹⁹ These females had the same incidence of asthma as a control group, although asthma was not as clearly defined in this study.

It seems unlikely that a disease of the modern age could account for the high frequency of this mutation, however this finding deserves further attention, possibly through further prospective study of screened individuals.

Why does ΔF508 cause cystic fibrosis ?

After the *CFTR* gene was cloned and the ΔF508 mutation identified, the initial hypothesis was that deletion of the phenylalanine residue would impede the nucleotide binding capability of the first fold.⁸ This theory was quickly reconsidered after a seminal paper describing the glycosylation of CFTR with and without the ΔF508 mutation.²⁰ It appears that

this mutation does not alter the chloride channel ability of CFTR, rather it corrupts the normal intracellular processing of this protein.

DEFECTIVE GLYCOSYLATION

Cheng and his colleagues examined the glycosylation of CFTR protein in immortalised cell lines.²⁰ They found two distinct 'states' of glycosylation, suggesting a maturation process from one to the other. The larger, 'mature', glycosylated protein did not appear in cells with the ΔF508 mutation suggesting an 'arrest' of the normal processing of CFTR. Full glycosylation of normal CFTR requires energy, in the form of ATP, but appears to confer stability to the protein which enables transport to the apical membrane via the Golgi apparatus.²¹ When CFTR contains the ΔF508 mutation (ΔF508CFTR), glycosylation is incomplete and rapid degradation of the abnormal CFTR occurs in a non-lysosomal area just outside the endoplasmic reticulum.^{22 23} Normal CFTR is also susceptible to degradation at this stage but, unlike ΔF508CFTR, a significant proportion enters the maturation process.²⁴

PROTEIN FOLDING

One group synthesised a polypeptide consisting of the 67 amino acids that surround the ΔF508 mutation and were able to demonstrate the complex folded structure of this region.²⁵ They suggested that ΔF508 may interfere with this folded structure and arrest the normal maturation of the protein.

Recognition of the importance of protein modification after gene translation has increased substantially over the last decade.^{26 27} During or immediately after translation in the endoplasmic reticulum, peptides undergo a series of modifications. Housekeeping proteins (termed 'chaperones') have been identified with this process.^{28 29} Two such chaperones, hsp70 (one of a family of proteins first recognised in stressed cells, heat shock proteins) and calnexin, have been associated with the CFTR protein.^{22 30} Calnexin is a transmembrane protein located across the endoplasmic reticulum and hsp70 is restricted to the cytoplasm. Normal CFTR is complexed with these chaperones in the endoplasmic reticulum but, during the maturation process, dissociates itself and is transported to the Golgi apparatus. In contrast ΔF508CFTR remains complexed to these chaperones and is rapidly degraded in the pre-Golgi compartment. It remains unclear whether aberrant binding with chaperones causes the arrested maturation of ΔF508CFTR or the arrested maturation (for example a folding defect) causes aberrant binding. In either case pharmacological manoeuvres targeted at the specific CFTR-chaperone complexes would be worth pursuing.

Correction of the trafficking defect

The inability of ΔF508CFTR to make its way through the cell is described as a trafficking defect (see fig 2). The functional potential of ΔF508CFTR can be demonstrated by experiments that overcome this defect.

Figure 2 Transport of CFTR through the cell.

It is now well established that the ion transport defect of cystic fibrosis cells in culture can be corrected by gene transfer incorporating DNA for normal CFTR.^{31, 32} These cystic fibrosis cells then show normal chloride conductance in response to cAMP. Surprisingly, however, when DNA coding for ΔF508CFTR was incorporated into non-human cell lines, cAMP mediated chloride conductance was also generated, albeit at a reduced level.^{33, 34} This appeared contradictory, however it soon became apparent that these non-human cell lines may have different pathways for processing CFTR. Protein folding mechanisms are known to be temperature sensitive³⁵ and the primitive cell lines, in which ΔF508CFTR appears to function as a chloride channel, exist at lower temperatures. The effect of temperature reduction on ΔF508CFTR expression was investigated. Mammalian cells, expressing ΔF508CFTR, were incubated at 26°C for two days. This led to the appearance of fully glycosylated protein and, more importantly, the demonstration of cAMP mediated chloride conductance.³⁶ This finding has since been repeated in cystic fibrosis airway epithelial cells (that endogenously produce ΔF508 CFTR). CFTR-type chloride conductance was demonstrated after incubation at 25–27°C for 24 hours.³⁷

These observations are important and demonstrate that ΔF508CFTR can function as a chloride channel if the intracellular trafficking defect is overcome, by, for example, temperature reduction. Several studies support this. Recombinant synthesis of the first nucleotide

binding fold with the ΔF508 mutation does not alter its ability to bind ATP.³⁸ 'Patch clamp' experiments have shown that ΔF508CFTR in the endoplasmic reticulum membrane functions as a chloride channel³⁹ and purified ΔF508CFTR protein, inserted into artificial membranes, has as much chloride channel capability as normal CFTR.⁴⁰

TRANSGENIC ΔF508 MOUSE MODEL

The generation of a transgenic mouse model, homozygous for the ΔF508 mutation, has enabled the 'ex vivo' examination of this temperature sensitive phenomena in tissues, as opposed to individual cells.⁴¹ Epithelial tissues, dissected from the distal colon were maintained at temperatures of 25–30°C for periods of up to 18 hours. Although the tissues maintained their ion transporting capabilities, no significant cAMP mediated chloride secretion was demonstrated (KW Southern, AW Cuthbert, unpublished data). This was disappointing. However, when tracheal epithelial cells from the mice were examined by a halide efflux fluorescence assay (a method for detecting evidence of CFTR function from individual cells) CFTR-type function was evident at 25°C but not at 37°C.⁴¹

Novel therapeutic strategies

Discovery of the CFTR gene has focused attention on gene replacement therapy and a number of early clinical trials have been initiated.^{42, 43} An alternative approach, however, is to utilise the functional capability of the ΔF508CFTR by overcoming the trafficking defect. Such a strategy, if successful, would be applicable to over 90% of people with cystic fibrosis.

In cultured cells, a non-specific stimulus generating overexpression of ΔF508CFTR resulted in small amounts of functional ΔF508CFTR reaching the plasma membrane.⁴⁴ The provisional results of a clinical trial of phenylbutyrate (a non-specific stimulator of gene expression) were reported at the 1996 North American Cystic Fibrosis Conference (PL Zeitlin). They suggested partial correction of the cystic fibrosis ion transport defect in patients with ΔF508. The final results of this trial are awaited with interest, however preclinical data suggest that a more specific and stronger promoter of gene expression will be needed if this is to be a feasible approach.

A number of chemicals, including glycerol,⁴⁵ have been shown to facilitate presentation of ΔF508CFTR to the cell surface membrane. Two novel compounds may have therapeutic potential. The xanthine derivative, CPX (8-cyclopentyl-1,3-dipropylxanthine), selectively activates chloride efflux from cells expressing ΔF508CFTR.^{46, 47} CPX is an A1 receptor antagonist, but is the only member, to date, of this class of drugs shown to exhibit this specific property. CPX has no effect on cells expressing normal CFTR. Its intrinsic lack of toxicity makes it a compound that deserves further investigation.

A group of compounds, termed heterotrimeric G proteins, have been linked with the

regulation of plasma membrane proteins through presentation of vesicles to the cell surface.⁴⁸ Inhibition of one of this class, $G\alpha_{12}$, has been shown to stimulate exocytosis and the appearance of cAMP mediated chloride secretion in $\Delta F508$ CFTR cells.⁴⁹ These early findings again deserve further investigation and encourage the hope that a pharmacological approach may be able to overcome the $\Delta F508$ CFTR processing defect.

Summary

Over 90% of cystic fibrosis patients carry at least one $\Delta F508$ allele and approximately 50% are homozygous for the mutation. An intracellular trafficking defect prevents presentation of this mutated protein at the cell membrane. Once in the correct position, $\Delta F508$ CFTR can function as an ion channel.

The processes involved in post-translational protein modifications are being unravelled. Mutations that disrupt these processes may be responsible for a large number of inherited conditions. Pharmacological manoeuvres aimed at correcting trafficking defects may allow us to utilise the functional potential of these abnormal proteins. Transgenic animal models will have an important role in this research.

Gene replacement therapy is not the sole therapeutic end point of molecular medicine. As knowledge of the $\Delta F508$ mutation expands, further strategies will develop to overcome the molecular defect. These will have clinical significance to most patients with cystic fibrosis.

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