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Cystic Fibrosis: Channeling the Discovery of CFTR Mutations

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Summary

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a protein forming chloride channels in the membrane of epithelial cells. It consists of two transmembrane domains (TMD), two nucleotide binding domains (NBD), and an R domain. The channel is first activated by the phosphorylation of its R domain. ATP then binds to CFTR’s NBDs for the channel to conduct Cl⁻ out of the cell. Mutations in CFTR can cause misfolding and prevent adequate transportation of Cl⁻. The most common cause of the disease is the deletion of phenylalanine 508 of CFTR. When ΔF508 reaches the ER, it is recognized as irreparable and thus targeted for degradation, which causes the symptoms of CF. The third most common mutation, G551D, does not function properly at the membrane. Molecular chaperones such as Hdj-2/Hsc70, Hsp90, and HspBP1 may rescue CFTR misfolding. This has therapeutic value because once ΔF508 is sent to the membrane, it can function normally. There are many treatments for CF, including airway clearing techniques, antibiotics, and lung transplantation. Recent research has suggested that oligonucleotide insertion, curcumin treatment, and digotoxin treatment can reverse the ΔF508 phenotype.

Introduction

Cystic Fibrosis (CF) is a disease marked by an ineffective chloride channel that afflicts thousands of individuals every year, yet there is still no known cure. It was first diagnosed in the 1700s by kissing a child on the forehead and if their sweat tasted salty, the parents knew their child had the disease (Littlewood, 2002). Currently, there are many more definitive ways of diagnosing this fatal disease. In 1938, Dorothy Anderson described the symptoms of a child with cystic fibrosis in detail, such that they could be applied in a clinical setting. By examining family inheritance, in 1946, Anderson discovered that cystic fibrosis was a recessive genetic disease. In 1953, it was revealed that children with cystic fibrosis had increased sweat electrolytes and therefore, sweat tests became an accurate means for diagnosis. Neonatal screening began in the 1970s, allowing for earlier diagnosis of this disease. In 1988, the first and most common mutation associated with cystic fibrosis, ΔF508, was found on chromosome 7 (Littlewood, 2002).

Cystic fibrosis is an autosomal recessive disease caused by the mutation of the CFTR gene. In 1985, the CFTR gene was located at 7q21-34, on the long arm of chromosome 7, which was soon followed by the identification of the gene sequence (Welsh, et al., 2001). The CFTR gene codes for a protein which contains 1480 amino acids and has been named the cystic fibrosis transmembrane conductance regulator, also known as CFTR (Southern, 1997). A mutation in CFTR often leads to CF. CFTR is a chloride channel expressed on the surface of epithelial cells in the intestine, respiratory system, pancreas, and sweat glands. Currently, there are over 1000 mutations comprising four classes that result in CF; however, one mutation, the deletion of phenylalanine at position 508, accounts for over 70% of CF patients. The other mutations are very rare, and only 4 others have frequencies above 1% (Littlewood, 2002).

There are over 1,000 new cases of cystic fibrosis diagnosed each year, and about 90% are diagnosed before the child is 3-years-old. Cystic fibrosis is most common among people of European background, with carrier numbers of 1 in 25. Mortality rates have been dramatically altered since the 1960s. In 1969, the mean life expectancy was 14 years. In 2005, however, it was 36.8 years (McCulley, 2007).

CF is characterized by thick mucus secretions in the pancreas, lungs, and reproductive organs. These thick mucus secretions block the airways and cause chronic bacterial infection and degeneration of the lung tissue. Patient death is often due to recurrent infections. Other symptoms of CF are the destruction of the pancreas and sterility in males (Quekett, 2007). A common biological model for CF is a transgenic mouse model (Clarke et al., 1992). Recently, efforts have been made to map the entire genetic structure of the most common cause of CF infection: the Pseudomonas aeruginosa bacteria. This way researchers can identify specific genes that cause the infection and turn off the target genes when needed (CFF.org, 2007).

CF does not have a cure; the only available therapies currently address the symptoms of the disease. Patients that suffer from severe lung disease caused by CF have the option of lung transplantation (CFF.org, 2007). Another treatment option for CF is the use of airway-clearing techniques to loosen the thick mucus in the lungs, making it easier to expel by coughing. Antibiotics used to treat CF are administered orally, intravenously, or inhalation therapies. These are commonly used after airway-clearing techniques and have the unique ability to reach the airways quickly and easily. Gene therapy is not currently available treatment for CF, but offers considerable promise if a proper vector can be found. These treatments present patients only temporary relief from their symptoms, and there is ongoing research to find a cure by specifically targeting mutations such as ΔF508.

Cystic fibrosis is a very well studied disease; however, there is still much to be learned. Over the past few years, tremendous advances have been made in this field. To understand the cellular impact of CF, we must first recognize how CFTR functions normally. Mutations causing CFTR to misfold and function abnormally, or not at all, must also be identified. To correct the adverse effects of these mutations, regulation of chaperones is a useful strategy. Addressing all of these factors lead to improvements in therapy, though there is still no cure for the disease.

Normal Function of CFTR

The key to discovering how mutations can affect CFTR is to first identify the channel’s normal functioning. CFTR is a protein found in the membrane of epithelial cells that acts as a chloride channel, moving negatively charged chloride ions out of the cell (Sheppard and Welsh, 1999). The transport of chloride is essential in maintaining constant cell volume. In
a process called RVD response, the chloride channels are activated allowing chloride to flow passively through the channel with water to restore homeostasis. CFTR is found in cells comprising many organs including the lung, pancreas, liver, or any other systems that require fluid mucus for their function. The water that is transported along with the negatively charged ions hydrates the cellular secretions in the aforementioned organs, aiding in fluidity (Anderson, 1992). In normal lungs the stabilization of NaCl concentration is aided by the passive transport of water into and out of the cells, producing a low salt content in the surface liquid coating airway epithelial cells (Dorin, 2004). This low salt environment facilitates the growth of naturally occurring antimicrobial peptides that fight off bacterial infections.

Consisting of two transmembrane domains (TMD1 and TMD2) along with two nucleotide binding domains (NBD1 and NBD2) (Riordan et al., 1989), CFTR is a member of the ABC transporter family due to its utilization of ATP hydrolysis in function (see Fig 1).

CFTR is activated when ATP binds to both its nucleotide binding domains causing NBD1-NBD2 dimerization (Vergani, et al., 2005). However, before ATP binds, protein kinase A (PKA) must first phosphorylate the R Domain of the protein, bridging the two homologous halves (the TMDs), and giving the protein configuration as follows: TMD1-NBD1-R-TMD2-NBD2 (Gadsby, et al., 2006). Negatively charged ions, such as chloride, are thought to accumulate near the positively charged regions of the pore until phosphorylation and ATP binding activates its opening, allowing the ions to flow through, down their concentration gradient.

Upon the intramolecular dimer interaction initiated by ATP binding to NBD1 followed by ATP binding to NBD2, a signal is transmitted through cytoplasmic linking domains to open the gate in the transmembrane domain. The activated channel is characterized by two open conductance states: O1 and O2 (Gunderson and Kopito, 1995). The O2 confirmation results in a 15% larger opening than O1, but the open CFTR confirmation only exists in this state 20% of the time.

The channel closes in two different ways, ATP dissociation or ATP hydrolysis, both of which result in a decrease of hydrogen bonding between the NBD domains (Ramjesingh, et al., 2003). Both cause a destabilization of the NBD dimer and consequently a lack of signal transmission through the cytoplasmic linking domains, causing the channel to close (Gadsby, et al., 2006).

The activity of the channel is characterized by the following directionality: C→O1→O2→C. Because the pathway is strictly directional, this gives further evidence that an outside source of energy (i.e. ATP) is needed to drive the gating asymmetry (Gunderson and Kopito, 1995).

Mutations in CFTR directly affect chloride conductance across the cell membrane, and without efficient anion flow, water movement slows and dehydrated mucus clogs and accumulates in organ ducts, fostering bacterial growth (Gadsby, et al., 2006). In addition to the decreased fluidity and increase in bacterial growth, because antimicrobial activity requires a low salt concentration and because CF surface fluid has a high NaCl concentration, Cystic Fibrosis epithelia fail to kill the thriving bacteria (Smith, at al., 1996). Failure of CFTR to function properly is often the effect of a number of mutations.

Class II mutation ΔF508: the Most Common Mutation in CF

There are four class mutations associated with CF, the most common form being the codon deletion of phenylalanine residue at position 508 from the NBD1 of the CFTR (Southern, 1997). The mutation of ΔF508 is a class II mutation and affects the two homologous halves of CFTR by inducing misfolding (Welsh, 2001). When the mutated CFTR protein reaches the ER, the quality-control mechanism of its cellular component recognizes that the protein is folded incorrectly and marks the defective protein for degradation. As a result, ΔF508 does not reach the cell membrane (Cui, 2006). Unlike CFTR, once wild type (WT) CFTR is synthesized it is transported to the ER and proceeds to the plasma membrane through ER export. Interestingly, the ΔF508 mutation does not alter the chloride channel ability of CFTR but rather corrupts the normal intracellular processing of the protein (Southern, 1997). Recently, scientists have concentrated on defining the class II mutation ΔF508 and, in the process, are discovering its functions, leading to better understanding of this fatal defect.

Protein Kinase CK2, CFTR, ΔF508: F508 Deletion Disrupts a Kinase-Binding Site

CFTR is part of a macromolecular complex in the apical membrane of epithelia comprising a number of protein kinases. The means by which ΔF508 CFTR alters the function of unrelated proteins is currently unknown. Through immunoprecipitation and Western blotting, Trehanar, et al. (2007) examined the relationship between the protein kinase CK2 and ΔF508. They found that CK2 associates with WT-
CFTR but not ΔF508 CFTR. Thus, CK2 appears to aid in the regulation of CFTR activity. Looking at the worm diagrams of hNBD1-7a-ΔF508 and hNBD1-2b- F508A Lewis, et al. (2005) found that the ΔF508 hNBD1 shows minimal conformational changes compared to mNBD1. Additionally, through surface topography, Lewis, et al. (2005) examined the worm diagrams of hNBD1-7a-ΔF508 and hNBD1-2b- F508A and found them to be dramatically different. The surface topography of hNBD1-7a-ΔF508 is altered at the site of the mutation, representing the presumed region of binding to the MSD1 of CFTR. When F508 is deleted, Val-510 (a side chain) is removed from its normal position (Lewis, et al., 2005).

Thus, the domains of hNBD1 with and without the ΔF508 mutation cause slight conformational changes. Although there are no significant differences in the folding properties of the two version of hNBD1, the slight structural change of hNBD1-7a- ΔF508 suggests that interdomain interactions are likely to be considerably altered by the ΔF508 mutation. These results demonstrate new insights into the molecular pathology of the predominant class II mutation, which may be of particular significance to efforts in discovery of treatment.

G551D: A Class III Mutation

A class III mutation affects CFTR function after the protein leaves the ER and is inserted in the plasma membrane. However, once it reaches the membrane, the chloride channel is defectively regulated. In the past few years, researchers have been focusing on the different ways the most common mutations act in a cystic fibrosis cell. G551D is the most common class III mutation and the third most common overall (Welsh, 2001); therefore, it is a good candidate for study. G551D is a missense mutation, which results in an exchange of asparagine for glycine at the 551 codon (McMorran, et al., 2001). This mutation occurs in 2-5% of CF patients. G551D is located on the apical membrane; however, it binds nucleotides less frequently and has reduced NBD1 ATPase activity (Derand, 2002). Therefore, the regulation of the chloride channel is defective. The G551D mutation was originally suspected to be located at or near the ATP binding domain. However, Howell, et al. (2000) found that the binding site was actually not near the binding domain. They realized that the substitution of the two amino acids caused the helix to loosen, therefore changing the shape of active ATP binding site (Howell, et al., 2000).

G551D causes a defect in the immunological response to inflammatory stimuli. McMorran, et al. (2001) infected mice with the G551D missense mutation using Pseudomonas aeruginosa. This is a bacterium that causes a lung infection to which CF patients are extremely susceptible. The research showed the decreased immunological response by comparing body weight, number of bacteria in the lungs, and the amount of pro-inflammatory mediators (TNF-α, KC and MIP-2) between G551D CF mice and mice with WT CFTR. The CF mice showed twice as much body weight decrease and a greatly increased amount of bacteria when compared to the WT mice. The CF mice also had a greater pro-inflammatory response with the TNF-α, showing a hyper-response to the bacteria. The amount of TNF-α decreased significantly after 48 hours, showing that there might be a feedback loop in which the diminished response causes further infection (McMorran, et al., 2001).
It was found that different mutations of CF would possibly require different types of treatment. The G551D mutation responded differently to three different agents that strongly influence chloride channels compared to the response of another CFTR mutation, G1349D. The three agents used were phloxine B, pyrophosphate, and 2-deoxy ATP. The phloxine B and 2-deoxy ATP had an effect on the G551D patients, whereas only the 2-deoxy ATP had an effect on the G1349D patients. The hypothesis given for 2-deoxy ATP working on both was that the binding sites for ATP are impeded by both of these mutations. 2-deoxy ATP was able to act as normal ATP and help both mutations function properly. The phloxine B increased the amount of chloride exiting the cell in the patients with the G551D mutation and in the patients with the G1349D mutation; however, it was not enough to save the cell in the latter (Cai, et al., 2006).

Another form of treatment for the G551D mutation would be the addition of genistein, a molecule that improves chloride channel activity by increasing the amount of time the channel is open (Suaud, et al., 2002). In WT CFTR cells, the normal chloride channel activity inhibits the epithelial Na+ channel, ENaC. However, when the chloride channel activity is low, ENaC works to stimulate it. G551D CFTR cells, ENaC and CFTR do not interact normally. Genistein was able to restore normal interactions between the two channels (Suaud, et al., 2002).

**Molecular Chaperones and Their Therapeutic Potentials**

In most cases, CF is caused by the mutation ΔF508 in the CFTR gene. The mutated protein is synthesized, but it is incapable of being sent to the plasma membrane through ER export due to misfolding. In this process, many molecular chaperones and co-chaperones are involved through either aiding the protein’s entrance into the secretory pathway or targeting the protein for degradation. Such chaperones include Hdj-2/Hsc70, Hsp90, and HspBP1. Depending on their functions, different chaperones play distinct roles in different stages of CFTR folding. Regulation of chaperone expression can be of great therapeutic value because once ΔF508 is sent to the plasma membrane it will be at least partially functional (Pasyk and Foskett, 1995). Hsp90 is an abundant chaperone in cells that plays an important role in preventing protein aggregation and helping proteins fold or degrade (Smith, et al., 1995). Thus, studies were done to find out whether Hsp90 can facilitate CFTR and ΔF508 CFTR folding. Hsf90 was found to bind to both CFTR and ΔF508 CFTR (Loo, et al., 1998). Ansamycin is a drug that inhibits the association between Hsp90 and its substrate. Thus, regulating the levels of Hsp90 can be used as a drug that inhibits that association between Hsp90 and its substrate. Then, whether ansamycin would also destroy the binding between Hsp90 and CFTR was investigated. In fact, no CFTR was found to be associated with Hsp90, indicating that the CFTR-Hsp90 complex was immediately perturbed when the drug was used. Once this was found, the effects of ansamycin on the synthesis of CFTR and ΔF508 CFTR was studied. Ansamycin inhibited the maturation of both forms of CFTR. Connecting these results, it can be concluded that if the association between Hsp90 and CFTR is disrupted, the maturation of CFTR cannot be achieved. Another hypothesis was that the perturbation of the Hsp90 and CFTR association would also accelerate the degradation of immature CFTR by proteasomes. This was investigated by looking at the effects of ansamycin alone compared to the effects of ansamycin and a proteasome inhibitor, lactacystin, on the synthesis of CFTR. As predicted, with just ansamycin there was a decrease in the amount of mature CFTR and lactacystin corrected its negative effects.

Concluding the data above, disrupting the association between Hsp90 and CFTR will inhibit the maturation of the protein and accelerate its degradation by the proteasome. Thus, regulation of Hsp90 can be used as therapy. Partner proteins of Hsp90 (such as p23) are also
being investigated to see if they play a role in facilitating Hsp90 in its function (Loo, et al., 1998).

**HspBP1 Inhibits CFTR Degradation and Stimulates CFTR Maturation**

There are two pathways in which CFTR can be directed while trafficking through the ER depending on whether it is folded properly or misfolded. When it is correctly folded, the CFTR protein will be sent to the plasma membrane and function normally. When it is misfolded, it will be recognized by specific chaperones and targeted for degradation. One of these chaperones is Hsc70 associated with CHIP (Connell, et al., 2001). By binding to Hsc70, CHIP induces the ubiquitination of the target protein, thus targeting it for degradation by the proteasome (Meacham, et al., 2001). There are other co-chaperones that form complexes with Hsc70/CHIP, which either enhance or inhibit its degradation activity. BAG-1 is a co-chaperone that promotes degradation when it binds to the complex. HspBP1 is another important co-chaperone and studies were done to find out whether it regulates the function of the Hsc70/CHIP complex. First of all, HspBP1 and CHIP were found to bind to Hsc70 at the same time but at different positions. Thus they do not inhibit each other's activity; instead they work in cooperation. When bound to the Hsc70/CHIP complex, HspBP1 was found to inhibit the ubiquitin ligase activity of CHIP, thus slowing the rate of degradation of CFTR. Thus, HspBP1 is another chaperone that, if it can be regulated, it will help the process of sending CFTR to the membrane (Meacham, et al., 2001).

**Innovative Treatment Possibilities**

Aside from common therapies such as lung transplantation, airway-clearing techniques, inhaled drugs, and antibiotics, recent research shows promise for other techniques addressing CF symptoms (CFF.org, 2007).

**Curcumin sends ΔF508 CFTR to the plasma membrane**

Curcumin is a major component of the Indian curry spice turmeric, which can act as a low-affinity sarcoplasmic/endoplasmic reticulum calcium pump inhibitor (Egan, et al., 2004). It was recently found to have possible therapeutic effects on ΔF508 CFTR. When BHK cell lines containing ΔF508 CFTR were treated with curcumin, the surface density of ΔF508 CFTR channels was found to significantly increase (Egan, et al., 2004). Using an iodide efflux assay with cAMP activation of the chloride channels, Egan, et al. (2004) were able to demonstrate that through its increased density at the plasma membrane, ΔF508 CFTR functions properly as chloride channels. Therefore it corrects the CF phenotype.

Results from co-immunoprecipitation suggest that curcumin interacts with Calnexin, a co-chaperone of Hsp90, which is involved in CFTR folding. Calnexin failed to coprecipitate with CFTR when the cells were treated with curcumin, suggesting that the interaction was inhibited. Thus, calnexin is thought to play a role in retaining ΔF508 CFTR in the ER (Egan, et al., 2004).

**Oligonucleotide Insertion Corrects ΔF508 CFTR**

Oligonucleotide insertion has also shown some promising therapeutic effects involving ΔF508 CFTR cell lines (Zamecnik, et al., 2004). This method of treatment intercepts the transcribed mRNA of the ΔF508 CFTR mutated gene, then corrects the deletion through insertion of nucleotide sequences that code for the missing phenylalanine. This corrected mRNA can then be translated into the WT CFTR protein, which is processed normally and transported to the plasma membrane. Zamecnik, et al. (2004) further show that the CFTR corrected by oligonucleotide insertion was functional at the plasma membrane by measuring the current after cAMP stimulation. Thus, oligonucleotide insertion prevents the development of the CF phenotype. While this treatment would not reverse the genotype of CF patients, it would be a preventative therapy.

**Cardiac Glycosides Suppress IL-8 Secretion**

Another fascinating advance in the realm of CF treatment is the use of cardiac glycosides, such as digitoxin, (Srivastava, et al., 2004). Cardiac glycosides are drugs that are usually used in the treatment of congestive heart failure. They work by inhibiting the Na’/K’ pump, which leads to an increase in the Ca” level, aiding in contraction of the heart muscle. This improves cardiac output and reduces any swelling of the heart. One of the major characteristics of CF is extensive lung inflammation, which is believed to be caused by hypersecretion of the pro-inflammatory protein IL-8. The ability of cardiac glycosides to reduce swelling led researchers to investigate their effects on ΔF508 CFTR cells.

All of the cardiac glycosides tested inhibited the secretion of IL-8, with digitoxin being the most efficient. Digitoxin blocks the phosphorylation of IKBα, an inhibitor of NF-κB activation, which stops the signaling pathway for hypersecretion of IL-8.

When treatment with digitoxin was compared to gene therapy, an interesting result emerged. Gene therapy significantly changes 58 genes, and 36 of those were equivalently and proportionately changed by treatment with digitoxin (Srivastava, et al., 2004). This suggests that digitoxin mimics the genomic effects of gene therapy on ΔF508 CFTR.

**Conclusion**

Cystic fibrosis is an autosomal recessive disease caused by mutations in the chloride channel, CFTR. The ΔF508 mutation of the CFTR gene, which was found to influence kinase-binding sites, accounts for the vast majority of CF. Concentrating on defining ΔF508 and its functions will lead to better understanding of this fatal defect and its molecular consequences. Another mutation, G551D, causes a shape change in NBD1. This makes it impossible for ATP to bind and activate the open-state of CFTR. Molecular chaperones can interact with CFTR in its folding and transport process in such a way that CFTR and its mutated form can both be sent to the plasma membrane and function normally. Some examples include the Hdj-2/Hsc70 pair which can facilitate the early biogenesis of CFTR, Hsp90 which can aid in CFTR maturation, and HspBP1 which can inhibit degradation and accelerate maturation of CFTR. Thus chaperones have significant potential in the treatment of CF. Aside from the common treatment techniques such as inhaled drugs, and antibiotics, the use of cardiac glycosides and digitoxin which can act as a low-affinity sarcoplasmic/endoplasmic reticulum calcium pump inhibitor and inhibit hypersecretion of IL-8, therefore preventing the inflammatory symptoms of CF. In order to assess the effectiveness of the aforementioned treatments, future clinical trials are needed.

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