NHLBI/ORD Workshop

Macromolecular Interactions and Ion Transport in Cystic Fibrosis

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Cystic fibrosis (CF) is a genetic disease caused by autosomal recessive mutations of the CF transmembrane regulator, CFTR. CFTR functions in the plasma membrane of epithelial cells lining the lung, pancreas, liver, intestines, sweat duct, and the epididymis. The primary problem in CF is that mutations in CFTR affect its ability to be made, processed, and trafficked to the plasma membrane and/or its function as a Cl⁻ channel and conductance regulator. Many proteins and processes normally interact with normal CFTR throughout its life cycle and mutant CFTR during the disease process. Understanding the function of these proteins and processes is expected to provide a clearer understanding of how normal CFTR is involved in salt movement and how mutant CFTR is handled by the cell and leads to the pathophysiology of CF. Recently, efforts to find therapies that correct defective CFTR have been intensifying. To facilitate our understanding of normal and mutant CFTR and the identification of new drug targets for developing novel therapies, a panel of experts was convened by the National Heart, Lung, and Blood Institute to explore the critical questions, challenges, and current opportunities to highlight new areas of research that would facilitate a integrated understanding of the processes and proteins that impact CFTR. The meeting highlighted the multiple pathways and interacting proteins involved in CFTR folding and biosynthesis, processing, and trafficking. A number of critical areas for future study were identified. Although these therapies are promising, a big question remains as to whether simply correcting defective CFTR will lead to significant improvement in patient health or whether the symptoms manifested in CF will require therapies in addition to those that target defective CFTR specifically.

Keywords: cystic fibrosis; CFTR; CFTR–macromolecular protein interactions; CFTR processing and degradation

Cystic fibrosis (CF) is a lethal genetic disease caused by autosomal recessive mutations of cystic fibrosis transmembrane regulator (CFTR) (1). The primary defect in CF is defective salt transport in plasma membranes of epithelial cells lining the organs of lung, pancreas, liver, intestines, sweat duct, and the epididymis (2). CFTR is a membrane protein that belongs to the ATP-binding cassette transport (ABC transport) family (1). It functions as a protein kinase A (PKA)– and adenosine triphosphate (ATP)–regulated chloride (Cl^-) channel, as well as a regulatory protein for the sodium channel (ENaC) and other channels (3). These well characterized functions of CFTR require its correct synthesis, maturation, and trafficking from endoplasmic reticulum (ER) and Golgi to the cell surface. Once at the plasma membrane, proper orientation with respect to other ion channels, regulatory molecules such as kinases and phosphatases, and the cytoskeleton are also critical for CFTR to function efficiently. Recently, a number of proteins have been identified that interact with CFTR, either in the biosynthetic process or in the final assembly of CFTR into a functional form. These molecules chaperone CFTR as it folds, participate in quality control of misfolded proteins, play a role in assembling CFTR into a complex with other proteins, and participate in regulating its activity, stability in the plasma membrane, and degradation of mature CFTR (4–13) (*see* Figure 1). One of the overall challenges in the future is to incorporate all of the processes and interacting proteins that impact CFTR into an integrated understanding of how CFTR functions in the human body.

Over 1,000 mutations in CFTR have been identified (14) that impact many aspects of CFTR processing, trafficking, and function. The most common mutation in CF, ΔF508 CFTR, is a misfolded protein, and is recognized as such by the ER, translocated to the cytosol, and degraded (15). A major fraction of the ΔF508 mutant neither matures and nor traffics to the plasma membrane. If some does reach the plasma membrane its half-life in the membrane is significantly less than that of wild-type CFTR (16). Misfolded proteins are recognized by the ER quality-control system and are targeted for degradation in ER-associated degradation (ERAD) pathways. Several proteins in the ERAD pathway have been identified that play a key role in processing of mutant CFTR proteins including Hsp70 and Hsc70 (17). Again, a challenge for the future will be to integrate the impact of proteins and processes involved in the ERAD pathway into a comprehensive understanding of how mutant ΔF508 is identified and processed for degradation in the ER and why it does not reach the plasma membrane.

Traditional therapies such as antibiotics, antiinflammatory agents, and pancreatic enzyme supplements are targeted toward ameliorating the consequences of defective CFTR function (18) that become evident in patients with CF, such as chronic lung infection, inflammation, and failure to thrive. But recently, efforts to find therapies that correct defective CFTR directly have been intensifying (19). For example, high throughput screening of chemical libraries has identified both activators and inhibitors of CFTR (20), and a recent clinical study involving AVV2CFTR has shown some clinical effects (21) such as improved pulmonary function. To enhance the development of new therapies aimed at mutant CFTR as the primary target, integrated information with regard to precisely how CFTR is folded, processed through the ER and Golgi, targeted to the plasma membrane or to degradative pathways, and regulated in the plasma membrane is critically important for rational drug design. Although therapies aimed at correcting mutant CFTR are promising, a big question

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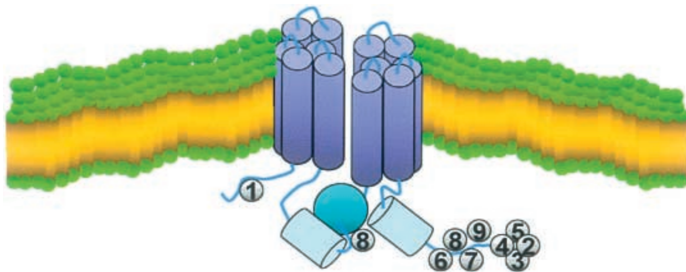

Figure 1. Proteins that have been identified and verified experimentally to interact with CFTR: (*1*) syntaxin 1A (4); (*2*) CAL = CFTR interacting ligand (5); (*3*) NHE-RF = sodium proton exchanger regulatory factor (6, 7); (*4*) E3KARP = adapter protein NHE3 kinase A regulatory protein (8); (*5*) CAP70 = CFTR-associated protein 70 (9); (*6*) Mu 2 (10); (*7*) AMPK = AMP-activated protein kinase (11); (*8*) CSP = cysteine string protein (12); (*9*) α-adaptin. Drawn by J. Cheng.

remains as to whether simply correcting defective CFTR will lead to significant improvement in patient health or whether the consequences of defective CFTR manifested over a long period of time in patients with CF will need to be corrected also. It is possible that therapies separate from those that correct defective CFTR will also be needed. Once defective function of CFTR is corrected in patients with CF, it will become evident how many of the consequences of defective CFTR function will have to be addressed separately.

HIGHLIGHTS

To facilitate the identification of new drug targets for CF in target areas, a panel of experts was convened by the National Heart, Lung, and Blood Institute (NHLBI) and the Office for Rare Diseases (ORD) on September 25–26, 2003 to explore the critical questions, challenges, and current opportunities in research related to macromolecular interactions between CFTR and many other proteins involved in normal CFTR function and misfunction in CF. The ultimate goal was to develop recommendations for new basic science research directions that will foster rapid translation of knowledge into the identification of novel drug targets and treatment strategies. The meeting highlighted the multiple pathways and interacting proteins involved in CFTR folding and biosynthesis, processing, and trafficking.

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF CFTR-BINDING ASSOCIATES

As CFTR progresses through the biosynthetic pathway from initial translation to final degradation, it interacts with many cellular proteins, some of which may vary between mutant and wild-type (*see* Figure 1). Assuming that some of these interacting proteins could make attractive drug targets and that the currently known interactions likely represent only a small fraction of cellular contacts, an important long-term goal is the identification of the “complete” cohort of interacting partners. Because this is likely to be a large effort, new technologies such as mass spectroscopy, genomics, and novel protein purification strategies should be employed in this effort. However, conventional studies will also be needed to characterize the role of identified proteins and to understand where all these interactions occur in cells. It is certainly likely that some interactions will occur in the biosynthetic pathway, others at the cell surface and still others in the endocytic/recycling pathway. Critical to our understanding of these multiple interactions is whether there is a regulated hierarchy to these multiple interactions and whether one set of interactions precedes another. Currently each novel protein that interacts with CFTR requires a significant amount of comprehensive study to understand its function. With an increasing number of interacting proteins and CFTR pathways identified, appropriate methods for integrating our knowledge will have to be developed. Although we have made some progress in identifying potentially interesting protein interactions, we need to develop more large-scale approaches to characterize these complexes and to compare the composition of protein complexes under different conditions and in different cell types and tissues. There needs to be an effort to assess interactions in different compartments, to determine temporal stoichiometry, and to define regulatory inputs that modify these interactions. Simplified screening of CFTR function is also needed in appropriate cell culture models that recapitulate how CFTR functions *in vivo*. Data from the genetic models will also need to be used in novel ways to characterize the function of multiple mammalian genes in polarized epithelial cells from CFTR-relevant tissues. Often critical reagents needed to speed the study of interacting proteins as well as to conduct localization studies are limiting or not available. Those that are available may not be significantly sensitive or specific. Additional resources for antibody generation and characterization would greatly facilitate these studies. Eventually antibody arrays may be useful for comparing complexes across cell types, in different tissues, and so on.

Efforts could be organized around obvious cellular themes, such as identification of early interactions involved in biogenesis, regulators of intracellular trafficking (vesicular trafficking), mediators of CFTR’s ion channel and conductance regulator functions, etc. These cellular themes are highlighted below.

CFTR Structure and the Pathways to Folding the Mature Protein

Recent progress has occurred in elucidating the structure of the first nucleotide domain of CFTR (22). Although important, it is clearly only a small first step and more will be needed to obtain the structure of full-length CFTR. Beyond this, understanding how CFTR folds *in cells relevant to CF* will be an exceedingly difficult and important area that will require detailed knowledge of CFTR structure as well as protein interactions involving numerous cellular chaperones and ER translocation machinery. Both areas will likely require development of new and innovative biophysical technologies. Lack of suitable approaches has been a major limiting factor to date, particularly regarding understanding the structure and folding of the full-length CFTR. A comprehensive picture of CFTR structure, folding, and maturation including the role of cellular proteins in modulating *in vivo* folding events and defects caused by inherited mutations such as AF508 should continue to be a major focus in future CF research.

Mechanism of CFTR Degradation and Maturation through the ER

Numerous cellular proteins often referred to as chaperones, are proposed to influence CFTR’s folding efficiency and degradation (23). Yet we still do not know even at a rudimentary level how they bind, what they recognize, and how their binding facilitates structural maturation. Important goals for future studies in this area are understanding the functional consequences of chaperone binding (or failure to bind), elucidating the kinetics of bind-

ing and release in relation to CFTR maturation, and identifying chaperone binding motifs on CFTR.

During CFTR biogenesis, a decision is made to divert immature and misfolded proteins into the degradation pathway (24). We currently have no idea of how or when this occurs. Understanding the balance between degradation and export of CFTR from the ER is important because potential therapeutic agents that enhance export may be particularly useful. However, the decision process will undoubtedly involve a close relationship between CFTR folding machinery and components of the ubiquitination pathway. For example, it is known that before degradation in the ER, ubiquitin is added to CFTR (25). Important issues in this area include the identification of ER quality control machinery specifically involved in CFTR degradation and the role of chaperone and co-chaperone proteins in degradation. Along these lines, it will be also important to identify CFTR-specific ubiquitin-conjugating enzymes and the mechanism by which ubiquitin-conjugating enzymes are recruited to CFTR (e.g., the relationship between CFTR, chaperones, and ubiquitination machinery).

Although current evidence indicates that CFTR degradation is a result of and not the cause of defective trafficking (24), the mechanism of CFTR degradation remains largely unknown, and it is entirely possible that certain steps in this process could present opportunities for intervention in CF or other related disorders.

Once CFTR is folded properly it must be exported from the ER for continued processing in the Golgi. Clearly, export of mutant CFTR from the ER plays a central role in CF pathogenesis (24). Understanding the proteins involved in selecting properly folded CFTR for export represents an area with rich potential for therapeutics. Export of wild-type CFTR from the ER requires COPII vesicle budding components, which are sensitive to mutations that disrupt normal coat protein complex II (COPII) assembly and disassembly (26). Understanding this key step will be important to understanding how COPII might regulate CFTR degradation by sorting CFTR into a specialized ER subdomains and subsequent entry into the proteasomal degradation pathway or processing to the Golgi.

Protein Interactions Involved in the Trafficking of CFTR through the Golgi

It is well known that CFTR matures to its fully glycosylated form within the Golgi (27). Compared with the ER, relatively fewer studies have focused on this important step. For example, it was suggested that compared with other proteins CFTR trafficking through the Golgi might be unconventional (28). However, this unconventional trafficking pathway may be confined to experimental cells and not to cells traditionally associated with CF. It certainly warrants further study. Recently, a novel protein, CFTR-associated ligand (CAL) containing two predicted coiled-coiled domains and one PDZ domain, has been identified that binds to the C terminus of CFTR. CAL is located primarily at the Golgi apparatus and modulates CFTR plasma membrane expression by retaining CFTR within the cell. In addition to regulating membrane expression, CAL also regulates the expression of mature CFTR by targeting mature CFTR for degradation in the lysosome (5, 29). CAL is only one protein involved in the Golgi processing of CFTR to mature protein and certainly more study will reveal many more relevant proteins.

Trafficking to and Recycling of CFTR at the Cell Surface

A number of proteins bind to CFTR and are involved as checkpoints for CFTR maturation to the cell surface. Once at the cell surface they are involved in stabilizing CFTR in the membrane. Processing of CFTR through the Golgi to the plasma membrane and subsequent stabilization in the membrane or entry into recycling compartments is important, especially for the ΔF508 mutant CFTR, which is known to have a shorter half-life in the plasma membrane (30). These differences could be attributable to failure of the mutant protein to recycle back to the plasma membrane in recycling endosomes (31). If this is the case, then understanding at what step(s) this process fails and what determines how wild-type CFTR is distinguished from mutant ΔF508 CFTR in the plasma membrane to account for these differences in residence time will be critical to successful drug therapies that are based upon increasing the density of mutant CFTR at the cell surface.

The Functional Form of CFTR

It is clear that purified CFTR functions as a Cl^- channel in lipid bilayers in the absence of other proteins (32). However, it is still unclear if CFTR functions as an independent unit or needs to collaborate with a host of other proteins in the plasma membrane to be fully functional in CF-relevant tissues. CFTR possesses a type I, C-terminal, PDZ-binding motif (33). Several PDZ domain proteins known to interact with CFTR at the plasma membrane include NHE-RF/EBP-50, and CAP70 (*see* Figure 1). The C-terminal tail of CFTR plays a potential regulatory role in modulating its Cl^- channel activity. Adding recombinant NHE-RF or CAP70 fusion proteins to the cytoplasmic side of CFTR *in vitro* increases the activity of CFTR (34). In addition, NHE-RF anchors CFTR to cytoskeleton stabilizing the cell surface CFTR (7, 35, 36). Both NHE-RF and its related protein, E3KARP, link PKA to CFTR (8, 35, 37). Moreover, NHE-RF was shown to link β2-adrenergic receptor to CFTR (38). All of this suggests that formation of such macromolecular complexes facilitates the efficiency of activation CFTR. Along these lines, it is known that CFTR regulates the function of other channels, including the epithelial sodium channel and the outwardly rectifying chloride channel, but exactly how this regulation occurs in not clear. For example, regulatory influences may occur by direct interaction or by some other mediation. Important to explore is the notion that the interaction of CFTR with other proteins may explain some of the other phenomena reliably associated with CF (e.g., abnormalities in glycosylation or in the inflammatory response).

It is well known that CFTR is regulated by PKC, PKA, and other kinases such as the metabolic sensor AMP-activated kinase (AMPK) (39). Data are available that some of these kinases may form complexes with CFTR and need to be closely associated for CFTR to be activated efficiently (35). Most of what is known involves wild-type CFTR. Clearly more information is needed with regard to whether this complex formation is defective in disease states.

Whether CFTR is a functional monomer or dimer is highly controversial. Much biochemical data, including immunoprecipitation of mixtures of different CFTR mutants and electrophoretic studies of CFTR using nondenaturing gels, as well as the genetic data that CF is autosomal recessive, have been advanced as evidence that the functional form of CFTR is a monomer (40). However, freeze-fracture electron microscopy suggested that the size of the CFTR channel is compatible with 24 membrane spanning domains, i.e., two CFTR monomers. Membrane crosslinking studies and some electrophoretic studies also suggest that CFTR exists in dimeric form (41). Additional information is needed to support the hypothesis that the most active form of CFTR is as a dimer, and that both monomers contribute to the function of the channel. Especially needed are structural data defining how CFTR is assembled into structural and functional unit.

Function/Dysfunction of Macromolecular Complexes in Determining the Composition and Amount of Fluid and Mucus Production and in Response to Infection and Injury

The regulation of the airway surface liquid layer is critical for normal mucociliary clearance (42), but how carefully the depth of the airway surface liquid is sensed and signaling pathways used by the sensors to control salt and water movement are not understood. If they exist, the sensors could be potential therapeutic targets.

Although it is well known that patients with CF have thick airway mucous secretions (18), the mechanisms involved in stimulating mucus production and altering the composition of mucus are not well understood. It is likely that altered mucus production and composition in the lungs of patients with CF influences the properties of mucosal surfaces and secretions that alter its ability to be colonized by bacteria. Thus, how mucus is altered in CF and the role of altered mucus in bacterial infection are still important issues that need to be addressed. It is clearly possible that bacterial infection may alter the interaction of CFTR with its interacting partners (43). Altered interactions may be either a normal response to bacterial infection which limits the damage caused by infection or an abnormality which exacerbates the pathologies exhibited by CF individuals. Thus identifying the key regulators of CFTR complex formation *in vivo*, understanding which components of the molecular complexes are affected by bacteria and how these complexes are regulated and rearranged in response to bacterial infection is important.

Also important is whether complexes involving CFTR and other proteins are cell type–specific in lung, pancreas, liver, male reproductive tract, and submucosal gland, and whether these differences determine how different tissues respond to mutant CFTR. Assessment of the impact of active/ongoing CF airway disease on CFTR regulation through macromolecular complexes may also help to explain why the airways of patients with CF have perturbed airway ion transport and chronic inflammation and why defects in CFTR lead to pancreatic disease and absence of the vas deferens. The severity of disease among patients with CF varies considerably, even among patients with the same mutation (44). This may be attributable to differences in genetic background among different individuals (45). One would expect that some of the proteins that interact with CFTR may influence disease severity if the interacting proteins play key roles in modulating CFTR function *in vivo*. Taking this one step further, it is also possible that some individuals who have an intact CF gene may have mutations or polymorphisms in proteins that interact with CFTR resulting in CF "like" syndromes with symptoms similar to CF.

Functional Consequences of Therapeutics Designed to Rescue ΔF508 and Other CFTR Mutants

CF is an autosomal recessive disease in which the heterozygote is without symptoms (46). Given this, it is safe to assume that an individual would be normal with at least half of the normal levels of CFTR. The question then for drug development is how much CFTR function is required to ameliorate the symptoms. It has been suggested that only a very small fraction, perhaps about 10%, of bronchial epithelial cells need to express wild-type CFTR to maintain a normal phenotype (47). Although it is often assumed that movement of ΔF508 CFTR to the apical membrane will correct the physiologic defect in patients with CF, this issue has not been examined in great detail. One caveat is that at the plasma membrane the ΔF508 mutant CFTR has less activity than wild type (48). Thus, the amount of ΔF508 needed to attain a normal phenotype may have to be much greater than for wild-type. In addition, once at the plasma membrane ΔF508 may also have to be activated to function properly making it necessary to both traffic mutant CFTR to the plasma membrane and activate it. Given the functional consequences that CFTR has on a wide variety of cellular proteins at the plasma membrane and the efforts currently underway to activate mutant CFTR and correct the trafficking defect (20), it is of major importance to determine whether appropriate interactions and their functional consequences are reconstituted by "corrected" mutant proteins. It is somewhat surprising how little effort has been invested in this area.

Development of New Techniques

It was also recognized that progress in the critical areas identified will require the development of novel techniques to study the physiologic implications of protein interactions. As there is an increasing numbers of molecules continuously being identified that impact upon CFTR and upon the pathophysiology of CF, the burden in time and resources on understanding the function of many more processes is increasing dramatically. Thus, there is a need to develop new high throughput methods of evaluating multiple proteins that impact CFTR and for testing for drugs that may effect macromolecular interactions.

Also, as CFTR moves through its lifecycle, interactions with its binding partners are likely to change both spatially and temporally. Hence, there is a need to visualize and manipulate protein interactions in real time with the goal of understanding the dynamics of CFTR interactions. To learn more about the physiologic role in the maintenance of overall heath we will need to determine how CFTR and its interacting proteins respond to normal environmental challenges, including the physiologic response to infection, with the goal of integrating the mechanisms involved in the host response to environmental challenges. Likewise, in relation to disease we will have to investigate the impact of CFTR mutations on the physiologic processes and on the manifestation of the processes that respond to the disease state and determine whether correcting defective CFTR function reverses the disease process and/or understanding what percentage of CFTR function is required to ameliorate disease. This puts an even greater need on the development of new realistic models of disease including well differentiated cell culture, or animal models.

SUMMARY AND PRIORITIES

Several critical areas were selected as those most critical to moving beyond our current knowledge. The basic consensus was that extending our *in vitro* knowledge of CFTR and its interacting partners to understand the physiologic consequences of CFTR interactions to tissue, organ, and whole body function and disease processes is most needed. One overarching need identified is to integrate both current and new information into real-time physiologic processes that are relevant to tissue, organ, and whole body function. Along the same lines another important goal is to provide an integrative understanding of the impact of mutant CFTR on the pathophysiologic processes and whether repairing mutant CFTR with various agents will reverse these processes.

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