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NM23 Proteins: Innocent Bystanders or Local Energy Boosters for CFTR?

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Abstract

NM23 proteins NDPK-A&B bind to the cystic fibrosis (CF) protein CFTR in different ways from kinases such as PKA, CK2 and AMPK or linkers to cell calcium such as calmodulin and annexins. NDPK-A (not-B) interacts with CFTR through **reciprocal** AMPK binding/control, whereas NDPK-B (not -A) binds directly to CFTR. NDPK-B can activate G-proteins without ligand-receptor coupling, so perhaps NDPK-B's binding influences energy supply local to a nucleotide binding site (NBD1) needed for CFTR to function. Curiously, CFTR (ABC-C7) is a member of the ATP Binding Cassette (ABC) protein family that does not obey 'clan rules'; CFTR channels anions and is not a pump, regulates disparate processes, is itself regulated by multiple means and is so pleiotropic that it acts as a hub that orchestrates calcium signalling through its consorts such as calmodulin/annexins. Furthermore, its multiple partners make CFTR dance to different tunes in different cellular and subcellular locations as it recycles from plasma membrane to endosomes. CFTR function in airway apical membranes is inhibited by smoking which has been dubbed 'acquired CF'. CFTR alone amongst family members possesses a trap for other proteins that it unfurls as a 'fish-net' and which bears consensus phosphorylation sites for many protein kinases, with PKA being the most canonical. Recently, the site of CFTR's commonest mutation has been proposed as a knock-in mutant that alters allosteric control of kinase CK2 by log orders towards calmodulin and other substrates after CFTR fragmentation. This link from CK2 to calmodulin that binds the R region invokes molecular paths that control lumen formation which is incomplete in the tracheas of some CF-affected babies. Thus we are poised to understand the many roles of NDPK-A and B towards CFTR function and, especially lumen formation, which is defective in the gut and lungs of many CF babies.

Introduction

Nucleoside diphosphate kinases (NDPKs/NME) are multi-functional proteins expressed as far apart as the amoeba *Dictyostelium* and modern humans. Humans have ten NDPK genes, *NME (non-metastatic) 1-9* and *RP2*, retinitis pigmentosa 2 whereas simpler organisms typically have about **one to** three isoforms.^{1, 2} NDPKs are classified into two groups (I and II) based on their sequence identities and capacity for high-energy phosphotransferase activity. Herein, we will refer to 'NDPK activity' as that role which is dependent on NDPK's internal phosphohistidine as a high energy source to effect change in another moiety. Thus group I genes encode proteins with NDPK activity and comprise NME1 – 4 (also known as NDPK-A, -B, -C, and -D; or Nm23-H1 - H4). These share higher sequence identity (58 – 88%) compared to group II, the latter additionally have low or no NDPK activity and share only about 22 – 44% sequence identity. Group I NDPKs (A - D) generate nucleoside triphosphates by covalently transferring the γ -phosphate from a donor nucleoside (or deoxy) triphosphate (NTP or dNTP, respectively) to an acceptor nucleoside (or deoxy) diphosphate (NDP or dNDP) in a ping-pong reaction using the high-energy phosphohistidine intermediate^{3, 4}. These NDPKs (perhaps as multimers) can also function as mammalian histidine protein kinases⁵ but, curiously, also possess 3'-5'-exonuclease activity⁶ which is important in DNA repair. This mini-review provides a summary of the current knowledge of the functions of two isoforms from group I in airway epithelia highlighting the role NDPKs might play in provision of energy for processes related to the protein whose mutation causes cystic fibrosis (CF).⁷

In the airway, ciliary clearance of inhaled contaminants entering the respiratory system prevents lung infections and is dependent upon transport of chloride and other anions into the lumen of the airways, accompanied by water and mucus movement. In CF, a disease mostly caused by just one in-frame triplet nucleotide deletion in the CF gene (the cystic fibrosis transmembrane conductance regulator, CFTR), lack of normal fluid and mucus transport results in an abnormal airway surface liquid (ASL) layer that accumulates thick

mucus with frequent lung infections from a host of unusual microorganisms.⁸⁻¹¹ Cell calcium plays a critical role in exocytotic mucus release and recently, CFTR has been shown to bind calmodulin, which might link anion transport to the viscid mucus found in CF airways.¹² The CFTR anion channel also recycles using endosomes to and fro from the apical surface of many epithelial cells and is a key hub that generates a macromolecular complex including both sodium and potassium channels, anion exchangers, transporters that export cAMP, and other regulatory molecules.¹³ We will return to this theme of CFTR as an input and output hub later in the review when we consider the molecular events leading to embryonic tracheogenesis where calcium-dependent exocytosis is used (in fetal life) to build a tracheal lumen.¹⁴

We have been interested in the constitutive protein phosphorylation of membrane proteins purified from airway epithelia and its relationship to ion transport and CFTR function for a number of years.¹⁵⁻¹⁸ Our work was initiated by our discovery that, provided sodium was absent *in vitro*, chloride concentration could act as a signal to purified apical membranes by regulating acid-labile phosphorylation of a number of soluble and membrane-bound proteins.¹⁹ In CF, it is established that mutation of CFTR causes a very low residence time and high degradation rate for the mutant (F508-deleted) protein which somehow enhances the function of epithelial sodium channels in a chloride-dependent manner.²⁰ Later, we identified that chloride-sensitive proteins such as NDPK, annexin A1 are phosphorylated on histidine residues; for annexin A1 this occurs near the conserved calcium binding domain of this regulator of inflammation and steroid signalling.^{15, 17, 21-23} Inhibition of NDPK by non-hydrolysable nucleoside analogues abrogated histidine phosphorylation of annexin A1.¹⁵ Other members of the annexin family are also implicated in CF disease and these will be discussed below **but we note that independent confirmation of the complex role of annexin A1 in CF pathophysiology has been reported in the last few years.**²⁴

CFTR is a member of the ATP-binding cassette (ABC) transporter/pump superfamily, which comprises membrane-anchored and 'scissor shaped' transmembrane proteins that use the

energy from adenosine triphosphate (ATP) cleavage to translocate a range of molecules across many different cellular membranes. ABC's are one of the largest and oldest protein families, with members found in all phyla. Over forty members are involved in essential processes in eukaryotic cells where their mutation causes disparate human diseases.²⁵⁻²⁷ They are multi-domain, and often self-assemble in lower orders in a multi-subunit manner comprising transmembrane domains (akin to scissor blades) bound to paired cytosolic clamps for ATP binding domains bearing at least one membrane-associated ATPase domain. Such domains trap two ATP molecules between their two cognate, inverted and opposed halves. CFTR channel function is driven by one ATP binding site acting as a non-hydrolysing 'degenerate' ATP holding 'bridge-like' domain with the other site permitting free hydrolysis that enables other domain shifts to facilitate pore access. The relationship between ATP binding and channel gating is a very complex process as reviewed recently by Zwick *et al.*⁷ However, these models take no account of the binding of CFTR-associated proteins that drive a cAMP or calcium-dependent CFTR activation^{12, 18} or to the possibility that CFTR might also become a (protease) split molecule that could recombine its N and C terminal fragments²⁸ in the membrane to create an array of different CFTR halves that add to the diversity of CFTR molecules in the membrane. It is accepted that hydrolysis of clamped ATP drives conformational changes in the translocator 'blades' structure, fuelling the transport of biological substrates across the cellular membranes.^{26, 27} Thus a typical structure of an ABC transporter comprises two transmembrane domains (TMDs), which are formed by six transmembrane α -helices with the pump energy supplied from two opposed cytoplasmic nucleotide-binding domains (NBDs)²⁶ bridged by two spatially separated ATP molecules. However, CFTR is a most unusual member of the ABC transporter family in that it is the only member that forms a transmembrane ion channel in which the ATP-driven changes in the cytosolic domains of the protein conformation result in the opening and closing of a distant gate at the entrance to the anion pore, allowing for the flow of pre-dehydrated anions (chloride, bicarbonate and other anions) down the prevailing electrochemical gradient^{29, 30}. In that sense, CFTR is a regulated slave to the direction of the

gradient of potential which means that anions can flow in or out of the cell on an 'as required' basis, even against a concentration gradient. Hence, CFTR is the equivalent of an 'if then else' statement in the central processing unit of a computer programme controlling a complex circuit bearing many other components and it is these regulators that form the focus of our review. This complexity idea provides a backdrop to the many roles of CFTR which seems to create causal actions at a distance from the channel through its many partners.

CFTR protein structure remains to be fully elucidated

The CFTR protein comprises a regulatory domain (R domain or R region), two transmembrane domains, TMD1 and TMD2, and two homologous (but non-identical) inverted and opposed nucleotide-binding domains, NBD1 and NBD2.^{25, 31-33} Electrophysiology suggests that the ion channel formed between the TMD domains of the CFTR protein opens when two different ATP molecules are bound to CFTR's two NBD domains. Conversely, closing of the channel gate follows a cycle of ATP hydrolysis, which leads to a different type of NBD1-NBD2 interaction and thus, to the termination of the flow of anions through the channel.²⁹ This complex gating cycle has a second 'if then else controller' in that the protein kinase-mediated phosphorylation of an unstructured cytoplasmic R- domain of CFTR is required to permit the channel-gating cycle. This unique (to ABC proteins) cytoplasmic R domain interacts with the two NBDs and is an integral part of CFTR protein that links NBD and TMD domains. The R region has multiple adjacent target sites for Protein Kinase A (PKA, a cAMP-activated protein kinase) phosphorylation which imparts a net double-digit negative charge to the protein.^{31, 34, 35} Thus, the central domain structure organisation of the single polypeptide 1480-amino-acid long wild type CFTR protein encompasses TMD2–NBD2–R–TMD1–NBD1.^{10, 35} **How such a system operates in an intact cell is not agreed with some recent data even suggesting that NBDs may be permissive for CFTR phosphorylation⁷.** We will return to the N-terminus and the C-terminus later when we (respectively) consider t-SNARES of the syntaxin family and protein kinases such as CK2 and AMPK **along with linkers from the DTRL motif at the end of CFTR to the cytoskeleton**

such as ezrin. Interestingly, the origins of the capture and insertion of the R domain in CFTR are unknown as no significant sequence homology with other proteins has been found to date.³⁶ PKA phosphorylation sites are highly conserved among species, and the R region has been shown to contain the majority of those sites.^{36, 37} This region may be likened to a net or web structure that is highly plastic with sub-regions of disorder and coil where transitions occur between these states when targeted by PKA and other protein kinases. One idea is that a randomly fluctuating R region becomes locally ordered with coil structures after regulation to permit the binding of other proteins to assemble a hub (or hubs). Thus, the R region acts as a molecular fly trap for passing cellular protein prey after protein phosphorylation imparts order through multiple negative charges. Thus CFTR does not act alone and a recent review of the data suggests that calmodulin is attracted to CFTR by this R region.¹² This R region may lie to the side of the structure based on atomic maps.³⁸ Interestingly, recent evidence from three-dimensional (3D) structures of both zebrafish (*Danio rerio*) and human CFTR from cryo-electron microscopy at a medium resolution of 3.7 Å in the absence of ATP reveals a) a funnel-shaped anion conduction pathway lined by positive charged residues, b) a single gate at the extracellular surface, and with c) the dephosphorylated regulatory domain located between (and not to the side of) the two NBDs preventing dimerization and channel opening.^{39, 40} The authors suggest that spontaneous disengagement of the R domain from its docking site in the intracellular vestibule likely leads to phosphorylation by PKA, correlating the channel gating with ATP hydrolysis rates. Comparative analysis of above conformations with zebrafish CFTR in its phosphorylated and ATP-bound form, shows large conformational changes after phosphorylation and ATP binding that promote channel function including NBD dimerization, disengagement of the R-domain from its inhibitory position and opening of the pore to the cytosol but not the extracellular space.⁴¹ Although these recent advances have provided such important and invaluable insights into CFTR function, further work remains to be done since CFTR function is also impacted by a complex of CFTR-associated proteins.

A key step in CFTR's regulatory functions is a consequence of targeting of PKA to CFTR. This approximates PKA, certain Na⁺/H⁺ exchanger regulatory factor isoforms (NHERF1 or NHERF2) and the cytoskeleton through the A kinase anchoring protein AKAP78 (ezrin, a members of the ERM protein family). The last two moieties play regulatory and structural roles in the stabilisation and relocation of many plasma membrane bound proteins. For example, ERM proteins act as a bridges-cum-molecular switches which when activated by PKC, PIP2, and Rho-GTPases promoting interactions with NHEFR1 so as to bind more tightly with CFTR. This may cause dimerization of CFTR and formation of a micro-domain where PKA can more efficiently activate CFTR.⁴² ERM proteins are also implicated (along with phosphorylation by kinase CK2) in optimally targeting sodium channels to their correct locations in the cytoskeleton of neurons. This is consistent with local substrate channelling of the constitutive activity of kinases such as NDPK and CK2 inside a new hub structure located near CFTR that may be shielded from the cytoplasm. Importantly, CK2 is also essential for sodium channel function by phosphorylating two different sites and others suggest that NDPK and CK2 may interact in vitro, but this remains to be confirmed.

Mechanism of action of the CFTR anion channel

It has been proposed, that pre-dehydrated, negatively charged chloride ions accumulate around the positively charged sections of the transmembrane domains that form the CFTR channel's pore. Ions such as chloride can then flow freely through the pore upon opening of the channel's gate.⁴³⁻⁴⁵ The course of events is initiated by the phosphorylation of the R domain of CFTR at multiple sites by an anchored PKA enzyme^{46, 47}, accompanied by the binding of ATP molecules to the two different ATP-binding sites on the inner faces of the cytoplasmic nucleotide-binding domains of the CFTR. ATP has been shown to be required for the opening of the phosphorylated CFTR channels. Channels do not open in the absence of ATP and the open channels close within 1 s of removal of ATP.^{12, 30, 47, 48} The binding of ATP to the nucleotide-binding domains of phosphorylated CFTR promotes the initiation of the channel gating cycle.⁴⁹ In most epithelial cells, the normal ATP concentration of ~ 2 mM

is thought to be sufficient to ensure channel activation. Additionally, it has been observed that the opening and closing of the CFTR channel is a cyclical process, with the activated channels showing typical opening durations of only hundreds of milliseconds.^{49, 50} According to a currently accepted model, this results in conformational changes, which in turn leads to the opening of the channel.⁵¹ This channel-gating cycle is terminated by the subsequent hydrolysis of the ATP promoted by the enzymatic activity of one NBD, upon which the ADP is released. Interestingly, ADP release is not reversible (ADP is not turned into ATP in the gating cycle under experimental patch clamp conditions *in vitro*). Moreover, the energy released during the ATP hydrolysis reaction is not used for the chloride 'pumping' or transport^{49, 52, 53}. It is instead proposed that the channel gating of CFTR can be promoted by non-hydrolysable ATP analogues, for example the AMP-PNP.^{54, 55} Confusingly, it has been demonstrated, that apart from having an ATPase activity ($\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{P}_i$), the CFTR enzyme may also manifest adenylate kinase activity ($\text{ATP} + \text{AMP} \rightleftharpoons 2 \text{ADP}$), which has been proposed to be essential to the normal CFTR activity in the epithelial cells of the lungs.^{46, 56, 57} These workers propose that both ATP and AMP interact with CFTR, albeit at separate binding sites, and that those nucleotides could mutually influence their interaction such that CFTR acts like an adenylate kinase.⁵⁸ This remains a controversial idea but is consistent with data linking the AMP activated kinase (AMPK) to the inhibitory control of CFTR channel regulation. Additionally, the cell essential protein kinase CK2, formerly casein kinase 2, is dominantly permissive over PKA in CFTR channel opening. For example, using two independent approaches with different *in vivo* preparations, coupled to data when human CFTR is expressed in different model systems, inhibition of CK2 prevents activated PKA from maintaining an open channel. Indeed, prompt closure occurs after about ninety seconds of CK2 inhibition in patch clamp experiments and despite adequate concentrations of ATP. Importantly, when a mutated and inhibitor-insensitive version of CK2 is present alongside CFTR, pharmacological CK2 inhibition can no longer overcome PKA activation of CFTR. These new recent data point to an inability to maintain an open CFTR

when CK2 (with active PKA) is inhibited suggesting that yet another control point may be operant during anion transport.⁵⁹

The thermodynamic basis of the CFTR channel gating cycle is still not understood, with contradictory opinions. Although the available data supports the assertion that dimerization and trapping of ATP between opposed and inverted NBD1 and NBD2 domains drives the process of channel opening, and that the ATP hydrolysis leading to the disruption of the NBD1-NBD2 interaction, results in channel closing, it is not clear how conformational changes in NBDs are translated into channel gating. Some studies have suggested that the process of the CFTR channel opening is temperature-dependent, while the closing of the channel was suggested to be only weakly dependent on ambient temperature.^{54, 60} Thus the event of the ATP binding, rather than ATP hydrolysis, has been suggested to be responsible for the opening of the CFTR channel.⁶⁰ However, the measurements of the contribution of the enthalpy and entropy to the energetic states of the open and closed state of the channel, which were obtained by the different groups (albeit using different subcellular cell purification methods and electrophysiological methods) are inconsistent and will require further elucidation. The channel gating of CFTR is not a thermodynamic 'near to' equilibrium reversible process, and thus, the non-equilibrium thermodynamic analysis may provide more consistent data and allow better understanding of the energy profile of the gating mechanism of CFTR.^{49, 61} It has been suggested, that the large activation entropy and activation enthalpy for channel opening may mean that the conformational transition at the gate may spread across domains while the channel is still closed and after dimerization of NBD domains is in place^{49, 61}. On the other hand, the small activation entropy for closing the channel could be more in line with the dissolution of a single phosphate bond in a trapped ATP molecule.⁶¹ This would suggest, that the channel closing is a process that does not involve the substantial conformational changes of the protein, and that indeed, this transitional step precedes the disruption of the NBD1-NBD2 dimer^{52, 61, 62}. Such a controller domain might include the C-terminal tail that is also implicated in the gating of the pore

because deletion of an acidic stretch in the tail close to the binding site for AMPK (see below) alters channel gating.⁶³ Interestingly, there is a site immediately adjacent to this acidic tail that is stoichiometrically phosphorylated by kinase CK2 at T1471 when peptides corresponding to this tail are used as CK2 substrates. Mutation of this T1471 site in full length CFTR is also implicated in the fragmentation of the protein **such that phosphomimics such as T1471D p attenuate CFTR fracture into N and C terminal halves.**^{28, 64} Thus at the time of writing, the molecular mechanisms by which the conformational changes due to the NBDs dimerization lead to the CFTR's channel opening remain clouded and in particular, how a supposedly remote tail domain of CFTR might 'talk' to the pore is a puzzle that will need a better picture of the full length of the CFTR structure that is currently lacking at atomic resolution. The combined data predict that the negatively charged region of the tail when augmented further by the addition of (CK2-transferred) negative phosphate charge will manifest an interaction with the pore. In this context, an earlier proposal that CK2 and NDPK can interact with each in vitro other remains an intriguing challenge for future work. This is especially interesting given that the transition between the monomeric and multimeric states of CK2 is itself dependent on ionic strength. **It is tempting to speculate that just as the R region of CFTR has been proposed to be a trap for proteins such as calmodulin,¹² perhaps multiple C-terminal interacting proteins whose expression might differ in different cell types⁶⁵ might also increase the functional tethering of CFTR in different cell contexts. Perhaps consideration should be given to the hierarchy of potential steric restrictions when multiple protein partners try to bind to the tail of CFTR.**

In a recent study with other ABC proteins, it has been demonstrated that the ATPase and the adenylate kinase activities are coupled such as in the ABC lipid A flippase MsbA, and in the LmrA and TmrAB family members. It may be that this feature could represent a general characteristic of the ABC transporters that might have been retained by CFTR.⁶⁶ Recent authors have shown, that the MsbA, a typical ABC pump, is able to catalyse both the ATP hydrolysis and the adenylate kinase reactions, and that the coupling could have a cyclic

character. Cycling may depend on concurrent physiological conditions, in which the ATPase activity would be most prominent under the normal physiological conditions, and the adenylate kinase activity would be most prominent under low ATP/high ADP cellular level. Coupling of the two activities (perhaps with AMP-kinase) would thus provide a mechanism that would allow flexibility for the ABC transporter to function under variety of physiological conditions, with adenylate kinase playing a role during physiological stress (such as the ATP depletion). In this context, it is important to remember that the cell metabolic sensor 'AMP-kinase' is a misnomer because this complex protein is both an AMP-stimulated and ADP-sustained kinase that binds to the C terminus of CFTR and phosphorylates the R region to keep CFTR shut at baseline. NDPK-A is central to this effect and PKA overcomes this blockade. Thus, it is possible that the coupled ATPase-adenylate kinase mechanism could be a general feature of ABC transporters, including the CFTR, and could represent a physiologically relevant feature of those proteins and protein complexes, representing their mode of functioning under the conditions of energy depletion.^{56, 58, 62} A key unexplained aspect of CFTR function is the discrepancy between the slow rates of ATP hydrolysis observed with purified CFTR and the fast gating kinetics of channel opening.⁶⁷⁻⁶⁹ We speculate that, as for many aspects of NDPK function (G proteins, ion channel and dynamin regulation, cell migration, Ras scaffold assembly, the fast turnover of NDPK (1000 per sec) could supply the missing link between the biochemistry and the electrophysiology.

Regulation of CFTR activity by the 5'-AMP-activated protein kinase (AMPK)

It has been established that phosphorylation of the R domain of CFTR is essential for the opening of the ion channel.⁵⁶ However, CFTR function is basally inhibited by the 5'-AMP-activated protein kinase (AMPK). It has been shown by yeast two hybrid and functional studies with interfering peptides, that the COOH-terminal regulatory domain of the catalytic $\alpha 1$ subunit of the AMPK interacts with residues 1420–1457 of CFTR⁷⁰, and that AMPK predominantly phosphorylates CFTR at Ser-768 in the R domain of the CFTR protein.⁷¹ The 5'-AMP-activated protein kinase is a principal metabolic sensor that responds to conditions

of nutritional demand and to the corresponding changes in the AMP/ATP ratio. AMPK exerts this function by inhibiting the ATP-consuming pathways in the cells⁷², and by regulating other energy demanding metabolic processes, such as glucose-dependent gene expression and cellular glucose uptake.⁷³ AMPK is a ubiquitously distributed (albeit with variable subunit composition) as a highly conserved heterotrimeric multi-substrate Serine/Threonine (Ser/Thr) kinase. It is comprised of a catalytic subunit (α 1 or α 2) and a regulatory subunit (β 1 or β 2), and a single AMP/ATP binding subunit (γ 1, γ 2 or γ 3). The multiple isoforms show differing tissue distributions. Low ATP to AMP ratio causes AMP to displace ATP from the γ subunit of AMPK, initiating a conformational change in the protein. That change allows the phosphorylation at Thr¹⁷² residue of AMPK by one of the upstream kinases followed by the activation of a catalytic α subunit.⁷⁴ Each of the γ subunits of AMPK has three AMP-binding sites, two of which are capable of reversibly binding either AMP or Mg²⁺-ATP and thus regulating the kinase activity of the AMPK protein, whereas the third AMP-binding site of AMPK binds AMP molecule irreversibly.⁷⁵ Under physiological conditions, whereby the concentration of the Mg²⁺-ATP is higher than that of the ADP, and much higher than that of the AMP, regulation of AMPK activity is also facilitated by a significantly higher affinity of AMPK for binding ADP than Mg²⁺-ATP. Although a rise in the local concentration of ADP and the corresponding decreasing ATP levels do not lead to the allosteric activation of AMPK, such conditions further enhance AMPK activity by preventing dephosphorylation of AMPK by phosphatases.⁷⁵ Working with the Hallows group, we reported that just one isoform of AMPK (alpha one and not two) and one isoform of NDPK (A not B) interacted with each other in the context of CFTR function. The data are supportive of a complex link between the activities of AMPK and NDPK-A which co-localise²³ (see below). This is compatible with our early data on chloride as a signal to phosphohistidine generation and perhaps there exists a latent functional inter-regulation between NDPK, that is part of an epithelial, chloride-sensitive, membrane-delimited phosphorylation system working with membrane-bound AMPK⁷⁶ in association with CFTR.

It is now evident that CFTR interacting proteins (CIP's) play an important role in regulating CFTR function.¹³ However, most CIPs have been identified under baseline conditions and as such are inhibitory to CFTR function.⁷⁷⁻⁷⁹ One such protein belongs to a family of t-SNARE proteins that are often found on endosomes. A model was proposed invoking the idea that at baseline, cell surface CFTR is held inactive (in complex with proteins inhibitory to CFTR function – e.g. syntaxin 1A, syntaxin 8; AMPK α ⁷⁷⁻⁷⁹) but after PKA is activated, additional component plasticity is induced which is permissive for channel opening.²² In this regard it was demonstrated that the calcium binding protein annexin A2 (anx 2) and S100A10 can form a functionally important complex with CFTR in airway and gut epithelial cells in a cAMP, PKA and protein phosphatase (calcineurin A) dependent manner.¹⁸ Interestingly, disruption of this complex significantly attenuates CFTR function in wild type cells. In CF cells bearing the common mutant form of CFTR, this PKA/CnA-driven anx 2-S100A10 complex with mutant CFTR fails to form.⁸⁰ The recent links between cell calcium and PKA and CFTR function have been reviewed elsewhere by Hanrahan, Kunzelmann and Mehta^{20, 81} (and cited by Forman-Kay¹²) and will not be discussed further here, but we note that calcineurin coupled to the calcium binding properties of the annexin family and calmodulin itself now firmly place this regulatory cation with the CFTR family of hub proteins.

Regulation of CFTR function by various isoforms of Nucleoside Diphosphate Kinase (NDPK)

NDPK-A (NME1, nm23-H1) and NDPK-B (NME2, nm23-H2) lie within group I of this histidine kinase family as two closely family members that share 88% sequence similarity. They are thought to exist in heterohexameric forms but our data suggest that they do not always create mixed moieties. Despite their close homology, the intracellular functions of NDPK-A and NDPK-B are markedly different and they do not co-localise to the same macromolecular complexes. For example, others have shown that NDPK-B, but not the NDPK-A, can bind and trans-phosphorylate the β -subunit of G-protein, thus increasing the activity of the α -subunit of G-protein.^{82, 83} This ligand-independent pathway promotes baseline cAMP

synthesis. NDPK-B has been implicated in the regulation of the G protein-coupled receptor TP β , and in the regulation of the activity of the calcium-dependent potassium channel KCa3.1.^{84, 85} The latter is also proposed to bind to CFTR.¹² The combined observations point to the distinct role of the NDPK-B isoform (which binds to NBD1 at residues 351-727), and histidine phosphorylation, in membrane-local signalling events that differ from the above interactions between NDPK-A, CFTR and AMPK. The role of CK2 remains unclear other than its proposed links to NDPK-A.

As with NDPK-B, the catalytic activity of NDPK-A also requires the 'self' or autophosphorylation event of the catalytic His-118 residue that lies in a water-excluding cleft common to both protein kinases. It has been suggested, that the residue Ser-120 of NDPK-A, could also be involved in the control of this self-phosphorylation process, perhaps via local electrostatic charge repulsion, as the negatively charged residue substitutions at Ser-120 inhibit autophosphorylation in NDPK-A and disrupt the AMPK-dependent inhibition of CFTR⁸⁶. That AMPK and NDPK might combine to retard the function of CFTR has an indirect link to faulty fatty acid metabolism found in CF disease. NDPK-A phosphohistidine generation regulates the key fatty acid regulator, the cytosolic enzyme ATP-citrate lyase⁸⁷, which then cleaves acetyl CoA from citrate exported from the mitochondria as a key step during cytosolic lipid synthesis. In parallel, AMPK regulates acetyl CoA carboxylase⁸⁷, a key enzyme generating the malonyl CoA that balances fatty acid biosynthesis versus fatty acid uptake by mitochondria. There are two possible mechanisms that could explain the role of the NDPK-A and AMPK in such regulation whether it be towards CFTR or in fatty acid metabolism. In the first "substrate channelling" model, nucleotides are directly supplied to AMPK-alpha-1 isoform by NDPK-A which controls local interconversion of various phosphorylated nucleotides. This would enable the AMPK to acquire local 'force fed' ATP and inhibit the CFTR Cl⁻ channel even in conditions when global ATP is depleted. The second mechanism stipulates that AMPK reciprocally modulates NDPK-A which acts as an intermediary between AMPK and CFTR in addition to regulating other signal mediators that

modulate CFTR activity.⁸⁶ Importantly, this NDPK-A/AMPK/CFTR interaction exists independently of the NDPK-B isomer⁸⁸ implying that CFTR may have different partners in different parts of the cell membrane with different functions. One possibility is that new membrane formation during membrane recycling needed for embryogenesis might be abnormally controlled after CFTR mutation. This idea is explored using lessons from fly genetics and tracheal lumen formation later in this review.

We have recently demonstrated that a fraction of NDPK-B, following cell stimulation translocates from cytosol to the membrane to form a functional complex with CFTR in the apical membrane of epithelial cells.⁸⁸ This is cAMP/PKA dependent process. We provided a range of biological and biophysical data consistent with the idea that NDPK-B interacts with NBD1 in CFTR via a motif involving amino acid residues 36-56 on the surface of NDPK-B. Thus NDPK-B, a key basal cAMP generator, is ideally placed to alter the function of NBD domains of CFTR, the dimerization of which drives ATP hydrolysis and thus the opening and closing of the channel. Since a concerted action of phosphorylation and ATP binding and hydrolysis is necessary to activate CFTR channel, it is proposed by these authors that cAMP binding may be another controlling event that takes place during this NDPK-B/CFTR interaction because cAMP enhances the binding of NDPK-B to CFTR in far Western experiments. The details of such a bridging interaction are still not understood but NDPK-B is a crucial element of the *in vivo* transport across the epithelial membranes via the regulation of the activity of both CFTR and related ORCC channels, once again stimulated by cAMP/PKA. Further research is necessary to elucidate the interplay of those activities in the CFTR under different physiological conditions. Since functionally, NDPK maintains pools of nucleotides for cellular processes central to energy utilization, association of NDPK-B particularly with CFTR's first NBD is interesting and suggests NDPK-B could play an important role in the provision and regulation of local nucleotide pools and or the energy required for NBD function. This may be relevant to the proposal by Dormer et al that CFTR

also has a cAMP binding site in its structure and the work of others suggesting homology between domains of CFTR and G protein structure.

CFTR and Calmodulin signalling.

The tracheal rings of CF babies are incomplete at birth and this may contribute to airway dysfunction. NDPK is an important player during tracheogenesis, along with cell calcium and the exocytotic cell machinery linked to endosome cycling. Forman-kay and colleagues have shown that cAMP and calcium regulation of CFTR coalesce through the acquisition of calmodulin binding to the R domain region and Kunzelmann, Hanrahan and Mehta have independently proposed a hidden role of cell calcium in CFTR function ^{12, 20, 81}. Tracheogenesis is a complex lumen generating process that has been unravelled using the power of developmental genetics in the fly.¹⁴ It is not common knowledge that flies have tracheas that feed the lumen of a branched tubular set of primitive airways that carry oxygen to mitochondria rich terminal tips in the fly body. The embryonic formation of a tracheal lumen first aligns and then hollows out adjacent epithelial cells that initially feel for each other using filopodial membrane extensions.^{89, 90} This blind attachment of membrane 'fingers' between cells may be likened to having magnets attached to the tips of each of the fingers of your two hands that are free to move in space. If the magnets are such that opposite poles are present on each hand, then the fingers will naturally gravitate towards each other. Now we have to imagine that the magnets then draw the long sides of the fingers together and consequently align each hand in a straight line. It was recently shown that adjacent tracheal cells put out finger like extensions from each side that fuse with the tips of their neighbouring equivalents during embryogenesis.^{90, 91} Next, microtubules attach to the fused tips mediating the local arrival of rab 11 and lysosome related organelles (LRO) together with Munc13-4/stac calcium binding proteins. This creates calcium spikes at the ER associated with fusion events that make a lumen by cellular hollowing. It is clear from parallel experiments in worms that tip migration and movement in other parts of development is dependent on NDPK function and Ras signalling through a scaffold protein called KSR.

Given that NDPK is dysfunctional in CF cells in that it is overexpressed, mislocalised and unable to generate phosphohistidine, a parsimonious explanation for tracheal ring malformation in CF babies might relate to an abnormal NDPK, CFTR interaction after CFTR mutation. These ideas, coupled to a link between the two calcium binding domains on Munc13-4/stac and their interaction with the t-SNARE role of syntaxin during late endosome fusion at the time of early fly tracheogenesis might be related to known interactions of the N-terminus of CFTR with syntaxins. Interestingly, a novel Munc13-4/S100A10/Annexin A2 complex has been shown to support acute Weibel-Palade body (WPBs) exocytosis by tethering WPBs to the plasma membrane via Annexin A2-S100A10 in endothelial cells⁹². Thus a wealth of disparate data from embryogenesis where cell tip migration, cell corpse disposal, autophagy and endosome fusion events have been recently linked to new membrane synthesis through fat metabolism are beginning to point to aspects of CF disease that are otherwise difficult to explain. A key hurdle is that those who research CFTR as a cause of CF do not share a research agenda with developmental biologists interested in tracheal formation, and in particular it is becoming clear that the embryo can reuse exocytotic events needed for mucus release during fetal lumen formation. For example, about 10% of CF babies have interrupted gut lumens at birth of such severity that they die without surgery and intensive care. We need to speak in a common language if those working on CFTR, membrane blocks in autophagy, endosome recycling and apical membrane function are ever to meet in a common research arena with those interested in phosphohistidine generation. For example, we suggest that new research might aim to link up the following ideas: (a) CK2 is log-orders overactive in CF cells bearing the common CFTR mutation;⁹³ (b) CK2 phosphorylates key residues on calmodulin located between two of its EF hands;⁹⁴ (c) NDPK controls dynamin as the terminal step in endocytosis and is important in cell homing during embryogenesis;^{95, 96} (d) AMPK and NDPK control adjacent supply points for the cytosolic availability of acetyl units needed for new membrane synthesis;⁹⁷ (e) CK2 phosphorylation controls both the localisation and function of sodium channels that become dysfunctional after CFTR mutation.⁹⁸⁻¹⁰⁰ These are some of the

known unknowns in CFTR and NDPK research that our model seeks to clarify. But how good is our model? In other papers in this series, using the worm NDPK model for example developed in Budapest, we observe a variable birth phenotype when just one NDPK gene is deleted in early embryonic life. In a related manner, not all patients will exhibit tracheal defects in childhood and only ten percent will develop defective gut lumens at birth needing surgery to avoid lethality. An important test of validity for any model of any disease is whether it can predict the existence of obscure complications. In our work, partly confirmed by others on other family members, we find that the first two members of the annexin protein family of calcium binding proteins are dysfunctional in CF^{21, 22, 101} alongside our reported NDPK defects. Combining our data with the known functions of annexin A2 in membrane hub assembly and signalling of exocytosis as proposed by the Gerke group⁹² for example, our model predicts platelet dysfunction in CF. The authors were unaware of this obscure CF literature at the time of using the model of ‘annexin-NDPK-opathy’ as a prediction tool. We were gratified to note that others have reported platelet pathologies and one paper¹⁰² has even proposed mean platelet volume as a biomarker for CF disease exacerbation. Thus we believe our approach linking poor CF phosphohistidine generation to an excess non-functional NDPK protein levels has predictive power for currently unexplained aspects of the disease phenotype.

In the title, we posed a question about the presence of NDPK A and B in the vicinity of CFTR. If NDPK-B is judged by its established roles in local energy boosting towards other proteins, then the next step must be to determine what is being boosted in the vicinity of CFTR. Whether that is CFTR itself or some other member of the multi-protein complex remains to be determined. For NDPK-A, our own data and that of independent scientists such as K Hallows⁸⁶ concur on a bi-directional regulator of NDPK in the context of AMPK's inhibitory role towards CFTR channel function. The other roles of NDPK-A and B in CF pathogenesis need to be determined but we note that NDPK-A has been proposed as a regulator of RAC in relation to the function of epithelial NADPH oxidases¹⁰³⁻¹⁰⁵ and that

NDPK-B is a core regulator of both ligand-independent G protein function and calcium activated potassium channel activity.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Acknowledgements

We apologise that we were unable, due to space constraints, to include in our review other protein kinases such as SYK that act as a further layer of regulation for CFTR. We thank Sam Miezi and Linda M Muimo for help with imaging illustration.

Figure Legends

Figure 1. Reducing knowledge gaps between NDPK, Annexins and CFTR.

Since this model was first proposed in 1998 for the F508 site, our new data shows that deletion of F508 residue from CFTR knocks in a new binding site for CK2 and mis-controls CK2 allosterically along with ATP concentration by log orders. It has recently been proposed¹⁰⁶ that many members of the Annexin gene family produce proteins that are virulence factors in pathogen host interactions. These include the commonest pathogen in cystic fibrosis (*Pseudomonas aeruginosa*) and notable viruses such as HPV type 16, CMV, Hepatitis C, and many others. In addition, it is becoming clear that pathogens can 'summon' cytosolic annexins to the plasma membrane by subverting cell signals and can even promote the extracellular expulsion of such proteins by unknown means. In this context, there seems to be an interaction between extracellular receptors for annexins related to the Formyl Receptor family and the recruitment of host neutrophils. It is well established that such neutrophils accumulate to excess in CF lungs and our work has reported that annexins such as annexin A2 and CFTR are found in the same complex.¹⁸ Since we have also reported that annexins can receive phosphohistidine in their core structure¹⁷ and that NDPK promotes p-His generation, then it seems a reasonable hypothesis that annexins, NDPK and CFTR are to be expected to co-locate. Our view is that this is a fertile area for future discovery given that annexins bring cell calcium sensing, membrane fusion events and modification of the host cytoskeleton during pathogen invasion into the picture. Since we have found that both annexin function and NDPK function are defective in CF cells, perhaps one idea for future work is that host defence might be enhanced when annexin and NDPK dysfunction is induced in a carrier of CF which in turn might explain why so many Europeans carry the common CF mutation. For example, we recently have related kinase CK2 directly to CFTR stability and CFTR function⁵⁹ through the C terminus of CFTR at a site proposed by Ostedgaard et al to be important in CFTR functioning.⁶³ That site near T1471 in the CFTR tail lies very close to the binding site for AMPK. Indeed we have also found that NDPK and

AMPK function towards CFTR are related to one another.⁸⁶ Recently, albeit in another tissue, annexin 1 and CK2 have been proposed to be related to one another¹⁰⁷ which provides independent confirmation that processes linked to cell injury, CK2 and annexin function are part of a common signalling pathway.

The star (★) refers to factors to consider in the interaction such as the role of fragments derived from this part of CFTR after its breakup in the membrane to generate a new deletion-induced F508del-based allosteric binding site for kinase CK2 that mis-controls both CK2 and substrate level phosphorylation towards proteins such as calmodulin and regulators of dephosphorylation including protein phosphatase 1 (PP1) and controllers of protein synthesis such as factors controlling chain initiation on the ribosome..

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