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Review

Influence of phosphorylation by protein kinase A on CFTR at the cell surface and endoplasmic reticulum

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Abstract

CFTR possesses a large cluster of strict dibasic consensus sites for phosphorylation by protein kinase A (PKA) in the R-domain and an obligatory dependence on phosphorylation is a hallmark of CFTR Cl^- channel function. Removal of as many as 11 of these sites reduces the conformational change in the R-domain and the degree of channel activation in response to PKA. However, until recently a completely PKA-unresponsive CFTR variant has not been reported, leaving open the possibility that the residual response may be mediated by associating ancillary phosphoproteins. We traced the residual PKA-catalyzed ^{32}P-labelling of the variant with 11 sites mutagenized (11SA) to distinct CNBr phosphopeptides within the R-domain. Mutagenesis of 4 additional monobasic sites in these segments produced a 15SA variant in which Cl^- channel response to PKA was abolished. Therefore, it can be concluded that ancillary phosphoproteins do not contribute to CFTR activation by PKA. Notably, however, the 15SA protein did exhibit a low level of constitutive channel activity not dependent on PKA, which might have reflected a down-regulating effect of phosphorylation of one or two of the 15 sites as suggested by others. However, this did not prove to be the case.

Since immature CFTR has been claimed to be active in the endoplasmic reticulum (ER), we also examined whether it can be phosphorylated in cells and what influence if any this might have on its susceptibility to degradation. Teleologically, activation by phosphorylation of CFTR Cl^- channels in the ER might be undesirable to the cell. Using various phosphorylation site mutants and kinase and phosphatase inhibitors in pulse-chase experiments, we have found that although nascent CFTR can be phosphorylated at the ER, this is without effect on its ability to mature and avoid proteolysis. Furthermore, we found that microsomes from cells expressing CFTR processing mutants such as ΔF508 do not generate Cl^- active channels when fused with planar bilayers unless maturation is promoted, e.g. by growth of cells at reduced temperature or other means. We conclude that the ER-retained mutant nascent chains which are incapable of maturation may be phosphorylated but do not form active channels. Stimulation by PKA of the insertion of CFTR containing vesicles into the plasma membrane as part of the mechanism of stimulation of chloride secretion has been reported, as has an influence of CFTR on the balance between endocytosis and exocytosis but these findings have not been universally confirmed.

Keywords: CFTR; Chloride channel; Cystic fibrosis; Phosphorylation; Protein kinase A; Endoplasmic reticulum

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Contents

1. Introduction

CFTR is unique among ABC proteins in several respects. First, it is the only one known to be itself an ion channel. Second, its ion channel activity is controlled by its phosphorylation state [1]. Although several other ABC transporters are substrates for phosphorylation by protein kinases, their primary functions do not seem to be dependent on their phosphorylation state. In contrast, under ordinary circumstances, the CFTR ion channel activity has an obligatory dependence on phosphorylation by cyclic AMP-dependent protein kinase (PKA). Indeed the response of CFTR to the action of PKA is the means by which hormonal stimulation of epithelial chloride secretion is mediated following elevation of cellular cAMP. Opening of the apical CFTR channel allows the cellular chloride concentration to fall, a change which is sensed by the sodium, potassium, 2 chloride co-transporter on the opposite, basolateral side of the cell [2]. These three ions thereby enter the cell from the blood side. Sodium ions are actively transported out again on the same side by the Na/K ATPase and potassium ions passively move out through a channel also in the basolateral membrane. The more negative luminal transepithelial potential caused by Cl^- exit through the CFTR channel promotes Na passage from blood through a paracellular pathway to lumen to complete this active secondary transport of NaCl [3]. This overall process was postulated and supported experimentally long before CFTR was discovered [4]. However, considerable data has been collected during the past decade indicating that CFTR provides the primary regulatory step in this transcellular routing of salt. CFTR also provides the apical conduit for chloride ions in several salt reabsorbing epithelia as best exemplified by the sweat duct [5]. In addition to PKA, CFTR also can be phosphorylated by several other protein kinases including protein kinase C, casein kinase II, cyclic GMP activated protein kinase, src kinase and possibly others. With the exception of PKC, however, which may influence the magnitude of the response to PKA, the physiological significance on the action of these other kinases on CFTR is unknown. The nascent precursor of the mature CFTR chloride channel can already be phosphorylated at the endoplasmic reticulum, an issue considered briefly in this article.

Although sites distributed throughout the cytoplasmic domains of CFTR may be phosphorylated, most PKA sites are clustered in a large one in the middle of the sequence.

2. Sites of phosphorylation

Initial inspection of the CFTR coding sequence revealed a plethora of consensus sites for phosphorylation by different kinases, especially PKA, PKC and casein kinase II [6]. These are particularly concentrated in the exon 13 sequence of the CFTR gene

PKA Phosphorylation Sites in CFTR

Kemptide: LRRASLG

CFTR: ...RKTSN.....RRNSI...KKQSF...RKNSI...
(422, 660, 686, 700)

RKFSI...RRLSL...RRQSV...RKTTA...RKVSL...RRLSQ...
(712, 737, 768, 788, 795, 813)

Fig. 1. Comparison of the peptide substrate (kemptide) commonly used in PKA assays with dibasic consensus sequences in CFTR; all except that at S422 are in the R-domain. Basic residues at positions −2 and −3 and hydrophobic residues at +1 with respect to the hydroxyl amino acids (highlighted) are underlined.

which contains nine strict dibasic consensus sequences for phosphorylation by PKA (Fig. 1) and several PKC sites. This region of the protein, termed the R-domain, was originally defined as the product of this single large exon; its actual structural and functional boundaries may vary considerably from those of exon 13 although these serve rather well to delineate the unusual concentration of PKA phosphorylation sites which have been found to be responsible for the tight control of channel activity. These sites were especially notable because there was already evidence of a defective cAMP-stimulated chloride conductance in CF epithelial cells [7]. Direct biochemical evidence that several of these sites were phosphorylated by PKA came from phosphopeptide analysis of a recombinant exon 13 produced in bacteria [8]. Activation of CFTR chloride channels by addition of the catalytic subunit of PKA to excised membrane patches from CHO cells transfected with full-length CFTR cDNA strongly suggested that direct phosphorylation of some of these sites might be responsible [9]. To gain more evidence of this, substitution of the serine residues at these sites with alanines by in vitro mutagenesis was performed. Removal of anyone of the nine dibasic R-domain sites individually by this means did not significantly diminish cAMP stimulated channel activity. However when multiple sites were mutagenized at the same time, activity was diminished. For example, simultaneous substitution of alanines for serines at residues 660, 737, 795 and 813 caused a major reduction in forskolin stimulated chloride efflux [10]. However, even when all nine dibasic sites in the R-domain as well as a tenth site N-terminal of NBD1 at residue 422 were mutagenized (10SA mutant; [11]) substantial PKA stimulated chloride channel activity remained.

To identify additional sites responsible for the response still exhibited by 10SA, it was phosphorylated with PKA using [γ-^{32}P]ATP as substrate. Cyanogen bromide digestion then yielded peptide fragments that could be identified using site-specific antibodies and deletion constructs. Much of the radioactivity localized to a CNBr fragment of ~5.8 kDa corresponding to a segment of the R-domain between residues 722 and 773 [12]. This fragment contains six serines of which two at positions 737 and 768 had already been changed to alanines in the 10SA mutant. The remaining four residues at positions 728, 742, 753, and 756 were individually substituted by alanine in the 10SA background. The 5.8 kDa fragment from all except the S753R version could still be phosphorylated by PKA. When tested in both efflux experiments and by patch-clamping the chloride channel response to PKA of cells expressing 11SA-S753R was reduced a further 40% compared to the 10SA.

However, two other CNBr fragments of the R-domain could still be labeled using [γ-^{32}P]ATP, indicating additional residual phosphorylation. These sequences (segments 647–721, and 774–837) contain three monobasic consensus sites for PKA phosphorylation at residues S670, T690 and T787. Replacement with alanine of these hydroxyl amino acids

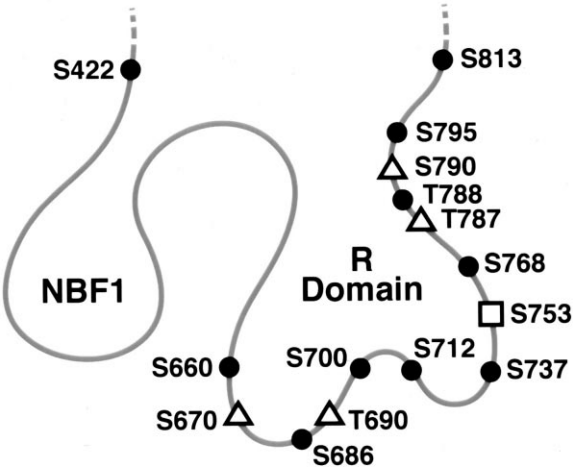

Fig. 2. Schematic depiction of 15 sites mutated to produce a PKA unresponsive CFTR (●) dibasic sites; □, △, monobasic sites identified by phosphopeptide analysis.

and a fourth one (S790) surrounded by several positively charged residues in the 11SA background yielded a 155A variant (Fig. 2; [13]). Despite the fact that it could still be weakly radiolabelled, no channel activity was elicited by PKA. Hence the removal of 15 PKA sites does render CFTR insensitive to activation by the enzyme, indicating that the entire PKA response may be mediated by direct phosphorylation of the protein without the need for involvement of ancillary phosphoproteins.

The achievement of a PKA insensitive CFTR channel by the removal of 15 sites implies that all or most of these may be involved in activation. However, it is well established that different sites quantitatively contribute very different proportions of the response [1,11,12]. It has been suggested also that different sites are qualitatively distinct such that phosphorylation of one or one set of sites may result in different channel gating properties than another [14]. In this view, instead of serving to ensure or to meter a single gated chloride permeability, the multiplicity of sites would generate channels with different biophysical properties. It has been reported that different levels of phosphorylation cause different modes of channel gating [15]. If indeed CFTR did evolve different functional PKA phosphorylation sites to accomplish diverse specific channel characteristics, it will be of great interest to learn the varied physiological roles subserved by each.

One study reported that mutagenesis of two of the dibasic sites, individually resulted in channels which when expressed in *Xenopus* oocytes could be activated by lower concentrations of the phosphodiesterase inhibitor, IBMX than wild-type [16]. This apparent increase in sensitivity to increased cAMP levels was interpreted as meaning that phosphorylation at either S737 or S768 diminished channel activation. However, in another study, restoration of serine residues at these two positions in the 15SA mutant returned substantial CFTR single channel response to PKA [13]. These different findings may mean that phosphorylation at these two sites has quite different effects on the protein depending on whether the other 13 serines can be phosphorylated or not. Alternatively, the differences in expression systems, activating stimuli and assays of channel activity may explain the apparent difference. If there are inhibitory as well as stimulatory actions of PKA phosphorylation on the R-domain, the physiological significance is not yet clear.

3. Mechanism of PKA activation

Since all of these sites except S422 are located in the R-domain, it must be crucially involved in channel activation. The results of several types of experiments are consistent with the idea that R-domain phosphorylation by PKA relieves a constraint from channel gating transitions which are driven by nucleotide interactions with the other major cytoplasmic domains, the NBDs [17–21]. Thus gating transitions cannot occur in the presence of MgATP without R-domain phosphorylation [1], deletion of the R-domain [17] or replacement of the hydroxyl residues at PKA sites with carboxylate residues to mimic the negative charges of the phosphoryl groups [18]. Both of the latter manoeuvers however produce only a minimal degree of constitutive activity. With glutamate residues at eight of the nine dibasic sites in the R-domain, the low spontaneous gating rates can be increased to the same level as the wild-type by phosphorylation with PKA. Thus while carboxylates at the strong dibasic sites alone are only partially effective, they seem to contribute substantially when combined with phosphorylation at other weaker sites. Therefore it is reasonable to conclude that at least part of the mechanism of channel activation by R-domain phosphorylation is due to charge effects. Perhaps the increased negative charge disturbs other charge interactions involved in keeping the channel closed. There are many positively and negatively charged residues in the cytoplasmic loops separating the transmembrane sequences which contribute to the ion pore as well as in the R-domain itself. Overall the molecule has a very basic isoelectric point [22]. The introduction of multiple double negative charges by the phosphorylation at many sites would lower this appreciably, an effect which could enable NBD-controlled channel gating. It would also be consistent with the need for phosphorylation at multiple sites rather than a single one for activation.

In addition to a passive negative effect of an unphosphorylated R-domain, some experiments have indicated a more active positive role of the phosphorylated domain [20]. This could help to ration-

alize the fact that only partial activation is observed when the domain is deleted [17]. The R-domain undergoes significant secondary structure changes which may be involved in the activation process on phosphorylation by PKA (Fig. 3; [23]). While these changes have been measured directly by circular dichroism only in an isolated recombinant R-domain produced in bacteria, a major mobility shift of the whole protein like that exhibited by the isolated domain occurs in SDS-PAGE on PKA catalyzed phosphorylation (Fig. 4). This shift is opposite in direction from that which would be due to addition of negative charges and probably also reflects a major conformational change.

As already mentioned, R-domain phosphorylation and dephosphorylation are not directly coupled to channel gating transition but permit them to occur as ATP interacts with the NBDs. Several authors have suggested that PKA phosphorylation may increase the affinity of CFTR for ATP [24–26]. Experimentally, support for the notion is largely at the level of inverse correlations between the amount of PKA phosphorylation or the number of sites phosphorylated and the ATP concentrations required for channel activation [26]. Hence, while affinity for ATP may be altered this has not been directly measured under appropriate conditions and it is also possible that the molecular change coupling the binding event to gating transitions is dependent on phosphorylation state. If this were the case, the relaying of the impact of the nucleotide interactions with the NBDs to channel opening and closing would be analogous to the events believed to be responsible for transport by other ABC proteins. In CFTR the coupling might only occur when the restraining influence of the R-domain is relieved by phosphorylation. As yet there is only a single report of a small decrease in the K_m for ATP hydrolysis on PKA phosphorylation [25]. Hence it is still necessary to more extensively evaluate the effects of phosphorylation on direct measures of ATP binding and hydrolysis as well as on potential conformational changes or alteration in domain interactions. Such studies should reveal whether unphosphorylated and phosphorylated CFTR see ATP differently or see it in the same way but respond differently.

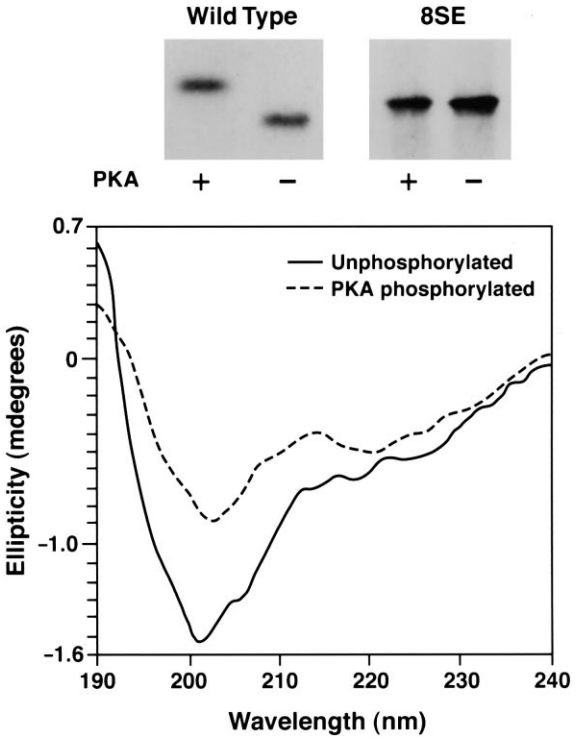

Fig. 3. Upper panels show large cathodic mobility shift caused by PKA phosphorylation on a recombinant R-domain polypeptide. Replacement of serine residues at 8 dibasic sites with glutamates (E) causes a partial shift not further influenced by PKA. Lower panel shows change in circular dichroism spectrum on PKA phosphorylation of R-domain.

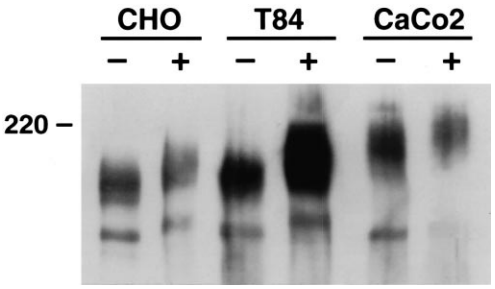

Fig. 4. Calyculin A (+) causes shift in mobility of both mature upper band and immature lower band of CFTR heterologously expressed in CHO cells and endogenously expressed in intestinal epithelial cell lines, T84 and CaCo2.

4. Effects of other kinases and protein phosphatases

While it seems clear that the action of PKA on CFTR is the primary mediator of hormonal stimulation of transepithelial chloride secretion, the molecule is also influenced by protein kinase C [9,27] and src kinase [28,29]). The major influence of PKC

seems to be to set the level of response to PKA rather than to act as a primary activating modification [27]. Identification of the exact sites involved has not yet been reported. Somewhat surprisingly, tyrosine phosphorylation by src kinase has also recently been reported to activate CFTR channels [29]. It is not yet clear, however, that this effect is entirely due to the phosphorylation of tyrosine residues on CFTR, although that does occur.

The CFTR channel is maintained in a quiescent state by phosphatase(s), some of which are membrane-associated and possibly in a complex with CFTR. In airway and colonic epithelial cells PP2C may be primarily responsible for inactivation of CFTR; PP2A or alkaline phosphatase are also able to reduce channel activity dramatically when added to membrane patches from CFTR expressing cells [30–32]. Other phosphatases which have been tested including PP1 and PP2B were found ineffective in these kinds of experiments. It is not yet clear whether or how the activity or accessibility of phosphatases acting on CFTR may be regulated.

5. Role of phosphorylation in nascent CFTR turnover

During CFTR biogenesis, the conversion of the immature nascent polypeptide already inserted in the ER membrane matures inefficiently and only one quarter to one third of the molecules become conformationally mature and competent to be exported from the ER, transported to the Golgi and on to the cell surface [33]. The remainder of the molecules are retained at the ER and degraded by the cytosolic proteasome and other proteases [34,35]. At least one report has claimed that nascent CFTR can generate a chloride channel in the ER membrane [36]. Teleologically, one might imagine that activation of chloride conductance at this intracellular location might be undesirable for the cell. Conceivably the extreme susceptibility to proteolysis may help to prevent this from happening. Nevertheless, a finite amount of immature CFTR must always be present in the ER membrane to serve as the precursor of the mature molecule. However, because the CFTR channel is so tightly regulated by phosphorylation, this small population present at steady-state in the ER membrane can be kept inactive as long as phosphatase activity exceeds that of PKA. As was shown in Fig. 4, this apparently is the case because the gel mobility shift reflecting the PKA-driven conformational change in the immature as well as the mature CFTR band is detected only when cells are treated with a phosphatase inhibitor, such as calyculin A. This indicates that indeed the nascent polypeptide may be phosphorylated at the ER in intact cells but that phosphatase activity normally keeps it in a dephosphorylated state. Hence, it is reasonable to think that rapid turnover and tonic phosphatase activity may both act to minimize CFTR chloride channel activity in the ER membrane.

Furthermore, it is of interest to ask if these two deterrents to channel activity at this location are related; that is, might phosphorylation of nascent CFTR contribute to its rapid turnover? This possibility seems reasonable because nascent chains of other ABC proteins not regulated by phosphorylation are not rapidly degraded in the ER. Furthermore, phosphorylation is a signal for ubiquitination and proteasomal degradation of many other proteins that need to be rapidly degraded to terminate their functions [37]. If this was the case with nascent CFTR, one might expect promotion of its phosphorylation to accelerate turnover. However, increasing its steady-state phosphorylation with calyculin A in fact does the opposite; both disappearance of the immature wild-type band and appearance of its mature product are inhibited (Fig. 5A). These effects are probably due to an inhibition by calyculin A of a step in the secretory pathway rather than to increased phosphorylation of nascent CFTR. This interpretation is supported by the fact that the phosphatase inhibitor also slows the turnover of two mutants in which the phosphorylation sites responsible for the mobility shift have been removed (Fig. 5B and C). Independent of the action of the inhibitor, the fact that nascent chain turnover and formation of its mature product are the same with wild-type and the 10SA variant (10 dibasic PKA sites mutagenized; [11]) argues that phosphorylation state is not a major determinant of nascent CFTR degradation or maturation. Strictly speaking these conclusions can be drawn from these observations only with respect to phosphorylation at dibasic PKA phosphorylation sites and those susceptible to calyculin A sensitive phosphatases. Since phosphatase(s)

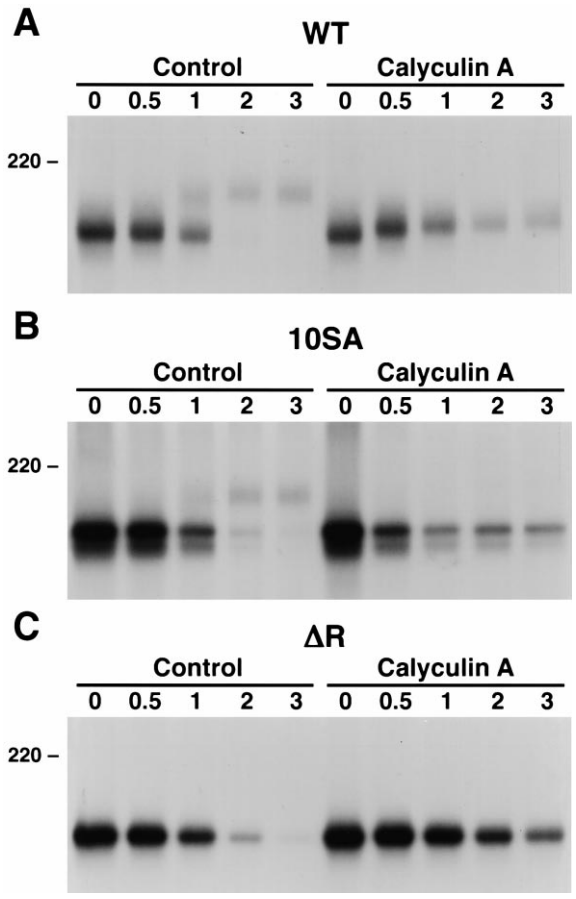

Fig. 5. Pulse-chase experiments showing time courses of disappearance of smaller immature precursor bands and appearance of large mature products in absence (control) and presence of calyculin A.

insensitive to this inhibitor such as PP2C contribute to CFTR channel inactivation [31,32], it remains possible that sites mediating these effects might accelerate degradation. However, because deletion of most of the R-domain (ΔR) or removal of all sites involved in PKA activation (15SA) still is without major influence on nascent CFTR processing this may be unlikely. The possibility that phosphorylation at other sites by other kinases may influence the fate of nascent CFTR has not been eliminated.

Aside from the issue of the potential channel activity of wild-type nascent CFTR in the ER membrane, it has been reported that the mutant ΔF508 CFTR nascent chain which never proceeds beyond this location has chloride channel activity [38]. This activity was detected by patch-clamping the ER membrane surrounding isolated nuclei from cells heterologously expressing ΔF508 CFTR. We have isolated microsomal membrane vesicles from similar cells for assays of channel activity after fusion with planar lipid bilayers. While wild-type CFTR channels are routinely recorded in this way [39], we failed to detect activity with vesicles containing ΔF508 CFTR unless its maturation is first promoted by growth of cells at reduced temperature or by mutating other sequences [40]. Hence, from these observations we conclude that nascent ΔF508 CFTR has the potential to become active if its maturation is promoted but that the misprocessed form normally present at the ER is not activated by phosphorylation.

6. Role of phosphorylation in the distal secretory pathway

Since increased cAMP stimulates exocytosis in several cell types including some chloride secretory epithelial cells, the possibility that CFTR may be involved in mediating this effect has been investigated in several laboratories. CFTR was found to be present in endosomes [41–43] and associated with clathrin-coated vesicles [44]. Bradbury et al. [45] reported that heterologous expression of wild-type CFTR in a pancreatic ductal cell line caused increased cAMP stimulated recycling and exocytosis of wheat germ agglutinin which had been internalized after binding to the cell surface. Endocytosis has been found to be inhibited by cAMP agonists in CFTR expressing cells and the protein itself probably undergoes continuous recycling between the plasma membrane and endocytotic vesicles [46]. However, there is not convincing evidence that phosphorylation of CFTR by protein kinase A influences its distribution between these two pools nor the insertion into or retrieval from the cell surface of these vesicles. Nevertheless some authors have shown that increased exposure of epitope-tagged CFTR at the surface of *Xenopus* oocytes [47] and MDCK cells [48] does occur on forskolin stimulation and suggested that this may be at least a part of the mechanism of CFTR chloride channel activation.

7. Summary

The CFTR chloride channel is extremely tightly regulated by its phosphorylation state. Addition of varying numbers of phosphoryl groups at different sites enables the channel to be gated by purine nucleotides at concentrations normally present in the cytoplasm. The level of activity is somewhat proportional to the number of sites phosphorylated enabling a metering of the chloride secretory response. When not stimulated, phosphatases maintain, both the mature cell surface protein and the immature nascent form at the ER in an inactive dephosphorylated state. Phosphatase inhibition results in increased steady-state phosphorylation of both. Phosphorylation of the nascent chain apparently does not influence its turnover either by proteolysis or conversion to mature protein. The ΔF508 nascent chain does not normally form a chloride channel that can be activated by phosphorylation but may do so if its conformational maturation is promoted by artificial means. The insertion into and retrieval from the plasma membrane of CFTR-containing vesicles may be influenced by phosphorylation but not necessarily that of CFTR.

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