Pflügers Arch - Eur J Physiol (2001) 443 [Suppl1]: S92–S96
DOI 10.1007/s004240100652

ARTICLE

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Regulation of the CFTR channel by phosphorylation

Published online: 13 July 2001

Abstract Cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels are regulated tightly by protein kinases and phosphatases. The regulatory domain of CFTR has about 20 potential sites for phosphorylation by protein kinases A (PKA) and C (PKC). The reason for this large number of sites is not known, however their conservation from fish to humans implies that they play important roles in vivo. PKA is an important activator, and its stimulation of CFTR is enhanced by PKC via mechanisms which are not fully understood. The physiological stimuli of CFTR are not known for some epithelia, and it appears likely that other serine/threonine and even tyrosine kinases also regulate CFTR in particular tissues. Phosphatases that deactivate CFTR have yet to be identified definitively at the molecular level, however CFTR is regulated by a membrane-bound form of protein phosphatase-2C (PP2C) in several cell types. Patch-clamp studies of channel rundown, co-immunoprecipitation, chemical cross-linking studies, and pull-down assays all indicate that CFTR and PP2C are closely associated within a stable regulatory complex. Understanding the regulation of CFTR by PP2C is a priority due to its potential as a target for pharmacotherapies in the treatment of cystic fibrosis.

Keywords CFTR · Cystic fibrosis · Chloride channel · Phosphorylation

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Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a low-conductance chloride channel that is activated by phosphorylation and gated by ATP binding and hydrolysis. The structure of CFTR resembles that of other ATP-binding cassette (ABC) transporters in having two membrane domains, each consisting of six transmembrane segments, and two nucleotide binding folds (NBF). CFTR activity is controlled by a central regulatory (R)-domain of approximately 200 amino acids. The topology and ATP-dependent gating of CFTR are unique among known ion channels, although its control by phosphorylation broadly parallels that of other channels in at least one respect: phosphorylation usually modulates the activity of ion channels by shifting their dependencies on ligands or voltage [18]. Recently, it has become clear that this may also apply to CFTR, since phosphorylation increases the apparent affinity of CFTR for ATP [19, 23, 40]. In the following we review briefly recent progress towards understanding the control of CFTR by phosphorylation and dephosphorylation, with emphasis on future directions. Extensive reviews of the literature on CFTR regulation have been published recently elsewhere [12, 37].

Phosphorylation by protein kinase A (PKA) is one major pathway for activation of CFTR

Most phosphorylation of CFTR occurs on its R-domain, which has nine dibasic (e.g., R,R/K,X,S/T) consensus sequences, five monobasic (e.g., R/K,X,S/T) sites, and numerous potential low-affinity sites for phosphorylation by PKA [17, 36]. Only about 6 mol of phosphate is added per mole of CFTR [31] or recombinant R-domain [10]. Sites known to be phosphorylated in vivo and in vitro have been reviewed recently [12]. Circular dichroism has demonstrated that the R-domain has relatively little structure [10, 29]. Its overall sequence among vertebrate species is divergent, nevertheless the consensus PKA

Table 1 Conservation among cystic fibrosis transmembrane conductance regulator (CFTR) homologues of the protein kinase A (PKA) phosphorylation sites on human CFTR that have been demonstrated in vitro

Human	422* RKT**S**	660** RN**S**	670* HRF**S**	700*** KRKN**S**	712** RKF**S**	737*** RRL**S**	753* RI**S**	768**** RRRQ**S**	795**** _RKV**S**	813** RRL**S**
Monkey	RKT**S**	RRN**S**	RRF**S**	KRKN**S**	RKF**S**	RRL**S**	RI**S**	RRRQ**S**	_RKV**S**	RRL**S**
Sheep	RKI**S**	RRN**S**	RRF**S**	KRKN**S**	RKF**S**	RRL**S**	R**S**N	GRRQ**S**	_RKM**S**	RRL**S**
Cow	RKI**S**	RRN**S**	RRF**S**	KRKN**S**	RKF**S**	RRL**S**	R**S**N	GRRQ**S**	_RKM**S**	RRL**S**
Mouse	RKH**S**	RR**SS**	RRF**S**	KRKN**S**	RKI**S**	KRL**S**	R**S**N	RRRQ**S**	_RKI**S**	RRL**S**
Toad	GNI**S**	RRN**S**	RRC**S**	KRK**SS**	RKF**S**	RKL**S**	R**S**N	RRRQ**S**	_RKM**S**	RRL**S**
Shark	RKMA	RRN**S**	HRF**S**	KRK**SS**	KKF**S**	RHF**S**	R**S**N	HRRQ**S**	_RKM**S**	RRL**S**
Salmon	NGQP	RR**SS**	RRV**S**	KRKQ**S**	RKF**S**	RKF**S**	RGN	QRRQ**S**	RKKL**S**	RPL**S**
Fundulus	NGQL	RR**SS**	RRV**S**	KRKP**S**	RKF**S**	RRF**S**	R**S**N	GRRQ**S**	RKKL**S**	RRL**S**

The *numbers* in the *first row* indicate the amino acid position in human CFTR; *, ** etc. relative level of phosphorylation

phosphorylation sites are conserved remarkably well from fish to humans, suggesting they have critical functions and must be located at appropriate positions (Table 1). When four serines that become strongly phosphorylated in vivo are mutated to alanines, CFTR channel activity in excised patches is reduced by about 50%. Interestingly, replacing S737 or S768 with alanines increases channel activity, suggesting that phosphorylation of these two sites inhibits channel activity.

The exact mechanism by which PKA phosphorylation increases CFTR channel activity remains obscure. The R-domain was thought originally to inhibit channel activity in a manner analogous to that of the inactivation ball of Shaker K^+ channels [35]. Direct evidence for this was provided subsequently by studies in which channels incorporated into planar bilayers were inhibited by addition of unphosphorylated R-domain to the cytoplasmic side of the channel [22]. Moreover, over-expression of R-domain in mammalian cells inhibits CFTR channels [30], while full channel activity is obtained when the two halves of CFTR lacking the R-domain are expressed in *Xenopus* oocytes [6]. An alternative (though not mutually exclusive) hypothesis is that phosphorylated R-domain activates CFTR, for example, by facilitating interactions between the NBFs, stimulating ATP hydrolysis, or accelerating ADP/ATP exchange. Evidence for such a catalytic function include the finding that the open probability of the ΔR mutant is much lower than that of wild-type channels activated by PKA [34], and that exogenously added phospho-R-domain increases the open probability of the ΔR mutant [40]. In summary, the basic mechanism of phosphorylation control remains unresolved, however the data are compatible with a model in which the R-domain inhibits the channel when unphosphorylated, and this inhibition is relieved and perhaps reversed by PKA.

PKA phosphorylation changes the conformation of bacterially expressed R-domain polypeptide (aa 595–831), as shown by circular dichroism and by its slower migration on SDS-PAGE gels [10]. These effects on conformation may be due in large part to phosphorylation of S737, since substituting alanine at this site reduces PKA's effect on electrophoretic mobility [4]. On the other hand, mass spectroscopy of the R-domain suggests that S768 is phosphorylated first, followed by 700 and 795, then 712 and 737, and finally 660, 670,753 and 813 [25]. The significance of such highly ordered phosphorylation is not known, and a different hierarchy might be obtained under different assay conditions. Nevertheless, it is reasonable to speculate that various phosphorylation sites may have different regulatory functions, in that submaximal phosphorylation of CFTR can increase open burst duration [14], decrease inter-burst duration [23], or both [41]. Hierarchial control by multiple kinases (see below) and ordered phosphorylation at multiple sites could modulate the gain of activation, enabling CFTR to be stimulated in intact cells by physiological levels of kinase activity. Indeed, most studies of CFTR have been carried out using cells treated with forskolin/3-isobutyl-1-methylxanthine (IBMX)/8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP), or using excised patches exposed to high concentrations of PKA catalytic subunit. Since physiologically important details of regulation may not be apparent during hyperstimulation, more studies with cell-attached patches and moderate levels of activation are needed.

Phosphorylation by protein kinase C (PKC) modulates activation by PKA

PKC does not activate CFTR substantially but seems to increase its responsiveness to PKA. This becomes apparent when PKA activation is examined at various times after patch excision [15]. PKA causes robust stimulation when added to freshly excised patches, but not when added 10 min after excision. We attribute the decline in PKA responsiveness to slow dephosphorylation of PKC sites by phosphatase activity within the excised patch because responsiveness could be restored by adding PKC to the bath in the continued presence of PKA. PKC alone does not stimulate channels after prolonged excision, consistent with its inability to cause a conformational changes in recombinant R-domain comparable to those induced by PKA [10]. Although the PKC phosphorylation is referred to as "constitutive" because it is present in unstimulated cells, this probably reflects our ignorance of regulation in vivo. In fact, most secretagogues stimulate both PKA

Table 2 Conservation of sites known to be phosphorylated on human CFTR by protein kinase C (PKC) in vitro

Human	660[a]*RN**S**	686***KQF**S**K	700[a]*KN**S**	790***KTTA**S**TR
Monkey	RN**S**	KQ**S**FK	KN**S**	KTAA**S**TR
Sheep	RN**S**	KP**S**FK	KN**S**	KTAT**S**TR
Cow	RN**S**	KP**S**FK	KN**S**	KTAT**S**TR
Mouse	R**SS**	KQ**S**FR	KN**S**	RTRT**S**IR
Toad	RN**S**	KQ**S**FK	KN**S**	_R**S**AVRK
Shark	RN**S**	NK**S**FK	K**SS**	RKT**S**VRK
Salmon	R**SS**	HQ**S**FR	KQ**S**	LQT**S**FRK
Fundulus	R**SS**	RQ**S**FR	KP**S**	QMQ**SS**FR

[a] S660 and S700 are also PKA sites

and PKC pathways under physiological conditions, and this coordinated control is probably not accurately reproduced when PKA catalytic subunit is added to excised patches. Further evidence that PKC regulation plays an important role in vivo comes from the fact that PKC sites on CFTR are almost as well conserved across species as are those for PKA (Table 2).

What is the mechanism of permissive regulation by PKC? cAMP stimulates more metabolic labeling of CFTR in intact cells after the cultures are briefly pretreated with phorbol ester to activate PKC [7]. This implies that PKC could act, at least in part, by enhancing the phosphorylation of CFTR by PKA, perhaps by exposing PKA sites that are otherwise inaccessible. However, such effects on conformation may be subtle since PKC phosphorylation has no measurable affect on the secondary structure of recombinant R-domain [10], and PKC pretreatment does not enhance overall phosphorylation of CFTR by low levels of PKA noticeably in vitro (D.A.R. Hinkson and J.W. Hanrahan, unpublished observations).

Other kinases also regulate CFTR

It should be emphasized that the physiological stimuli regulating CFTR are unknown in many tissues and may not always be PKA and PKC. Other G protein regulated serine/threonine kinases have been implicated in the sweat duct [33]. The tyrosine kinase $p60^{c\text{-}src}$ modulates PKA activation of CFTR in a manner reminiscent of PKC [11]. In other in vitro studies, $p60^{c\text{-}src}$ alone phosphorylated and activated CFTR strongly[16]. Activation by *Src* is unexpected because phosphotyrosines have not been demonstrated previously on CFTR, which lacks cannonical tyrosine kinase sites. Nevertheless, exogenous *Src* does phosphorylate CFTR and stimulates its channel activity in excised patches to levels comparable those evoked by PKA. Moreover, CFTR channels become spontaneously active when coexpressed in cells with constitutively active $p60^{v\text{-}src}$, and are further stimulated by treating cells with the tyrosine phosphatase inhibitor dephostatin. *Src* regulation does not involve PKA since exogenous *Src* can fully activate a CFTR mutant that lacks 15 potential PKA sites and is unresponsive to PKA. Comparing the effects of *Src* and PKA phosphorylation on conformation of the R domain might provide some insight into the changes critical for channel activation. The response to *Src* also raises the possibility that CFTR may normally be regulated by other tyrosine kinases, such as $p62^{c\text{-}yes}$ [24]. Finally, CFTR Cl conductance and cAMP responsiveness both increase in lymphocytes during the G1 phase of the cell cycle [5]. This may reflect up-regulation of CFTR expression or stimulation of CFTR by cell cycle-dependent kinases.

CFTR is dephosphorylated by membrane-bound PP2C; evidence for a regulatory complex

CFTR channels deactivate quickly when membrane patches are excised from Chinese hamster ovary (CHO) cells [39] or airway cells [2]. This rundown is presumably due to dephosphorylation of PKA sites because it does not occur if PKA catalytic subunit is present, and channels can be fully restimulated by exposure to PKA during the first few minutes [3, 39]. Rundown indicates that at least one of the phosphatases regulating CFTR is membrane-delimited and remains active in the excised patch.

In contrast to the spontaneous deactivation that occurs in patches from CHO cells, CFTR channels expressed in baby hamster kidney (BHK) cells often remain active indefinitely after excision. We have used these channels to study regulation by exogenous phosphatases [20]. Exposing CFTR to purified PP2A, PP2C or alkaline phosphatase reduces activity by more than 90%; however, PP1 and PP2B have little effect, despite comparable phosphatase activities in biochemical assays that employ phosphorylated myosin light chains as the substrate. PP2C deactivates CFTR channels more rapidly than PP2A or alkaline phosphatase and the time course resembles that of spontaneous deactivation (in the subset of patches that display rundown). Deactivation by exogenous PP2A, but not PP2C, is associated with shortening of the mean burst duration, suggesting that these two phosphatases may act on functionally-distinct PKA sites. Deactivation of CFTR-mediated short-circuit current across T84 epithelial cell monolayers (i.e. active Cl secretion) after wash-out of agonists is insensitive to calyculin A at high concentrations. Since PP2B dephosphorylates CFTR less efficiently than other protein phosphatases and there is no evidence that deactivation is calcium- or calmodulin-dependent, PP2C is left as the phosphatase most likely to be responsible for deactivating CFTR in intact cells. Bromotetramisole, a commonly

used alkaline phosphatase inhibitor, stimulates channel activity in intact cells and in excised patches [2], and inhibits all four types of protein phosphatases at concentrations used to stimulate CFTR [21].

Recent studies indicate that CFTR and PP2C exist in a stable complex, which facilitates down-regulation of the channel when cAMP stimulation is terminated. A monoclonal anti-CFTR antibody co-precipitates PP2C from (BHK) cells stably expressing CFTR, but does not co-precipitate PP1, PP2A or PP2B [42]. Conversely, a polyclonal anti-PP2C antibody co-precipitates CFTR from BHK membrane extracts. A specific association between CFTR and PP2C was confirmed by chemical cross-linking with dithio-bis(sulfosuccinimidyl propionate) (DTSSP), a hydrophilic, bifunctional reagent, which is cleaved by dithiothreitol after purification but before electrophoresis. Exposing CFTR-containing BHK cell lysates to DTSSP leads to cross-linking of histidine-tagged CFTR with PP2C, with both proteins appearing in the same elution fraction during Ni^{2+}-nitrilotriacetic acid (NTA) agarose chelate chromatography [8, 42]. Cross-linking of PP2C with CFTR is specific, since PP1, PP2A and PP2B do not co-purify with CFTR after DTSSP treatment. The association has also been observed recently in pull-down assays in the absence of cross-linker. So far only unstimulated cells have been studied, although it is conceivable that the association between CFTR and PP2C is itself regulated by PKA or other factors.

Future directions

Rapid progress is expected towards defining the interactions between CFTR domains and interactions of CFTR with other proteins. CFTR can influence the activity of many other membrane transporters and it will be important to learn the mechanisms of those apparently non-specific interactions regardless of whether they are considered true “functions” of CFTR. The influence of CFTR on other proteins such as the epithelial Na^+ channel (ENaC) may not be functions in the usual sense, but could still be crucial to the electrolyte and fluid transport abnormalities in cystic fibrosis [38]. In addition to transporters, the amino terminus of mature CFTR interacts directly with syntaxin 1A, a protein involved in vesicle fusion in neurons [27, 28]. CFTR competes with Munc 18 for binding to syntaxin 1A, and the binding of syntaxin 1A may be competitive with intra-molecular interaction between the R domain and a helical region near the amino terminus of CFTR (see [26]). It will be interesting to learn how these domain-domain interactions affect interactions of CFTR with kinases and phosphatases. The C terminus of CFTR has a motif recognized by the PDZ1 domain of the (50-kD ezrin-radixin-moesin-binding protein (EBP 50). Mutations and deletions at the C terminal tail of CFTR preventing interaction with this scaffolding protein may partially disrupt apical localization of CFTR in polarized epithelial cells (e.g., [9]). Other protein-protein interactions may influence the processing, targeting, or stability of CFTR (i.e. its half-life at the cell surface).

Another area of rapid progress is likely to be that of CFTR processing, aided by insertion of epitope tags, fusions with green fluorescent protein, confocal microscopy, and other tools of cell biology. Biochemical characterization of full-length CFTR protein [19] and individual domains has begun (e.g., [10, 29, 32]), and following recent successes in crystallizing nucleotide binding domains of the bacterial ABC transporters RbsA [1] and HisP [13], high resolution structures of CFTR domains may soon be available. A major hurdle for such studies remains the difficulty of obtaining sufficient amounts of CFTR in its native state, and reconstituting it in a way that fully preserves its activity.

Acknowledgements We thank Tom Irvine and Stephanie Fung for comments on the manuscript. This work was supported by grants from the Canadian Institutes of Health Research (CIHR), the Canadian Cystic Fibrosis Foundation (CCFF), and the National Institutes of Health (DK54075). Y.J. and T.Z. are CCFF fellows and J.L. received a CCFF studentship. J.W.H. is a Senior Scientist of the CIHR.

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