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ORIGINAL ARTICLE

Phosphorylation of cystic fibrosis transmembrane conductance regulator (CFTR) serine-511 by the combined action of tyrosine kinases and CK2: the implication of tyrosine-512 and phenylalanine-508

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Abstract The cystic fibrosis transmembrane conductance regulator (CFTR) harbors, close to Phe-508, whose deletion is the commonest cause of cystic fibrosis, a conserved potential CK2 phospho-acceptor site (Ser511), which however is not susceptible to phosphorylation by CK2. To shed light on this apparent paradox, a series of systematically substituted peptides encompassing Ser511 were assayed for their ability to be phosphorylated. The main outcomes of our study are the following: (a) Tyr512 plays a prominent role as a negative determinant as its replacement by Ala restores Ser511 phosphorylation by CK2; (b) an even more pronounced phosphorylation of Ser511 is promoted if Tyr512 is replaced by phospho-tyrosine instead of alanine; (c) Tyr512 and, to a lesser extent, Tyr515 are readily phosphorylated by Lyn, a protein tyrosine kinase of the Src family, in a manner which is enhanced by the concomitant Phe508 deletion. Collectively taken, our data, in conjunction with the notion that Tyr515 is phosphorylated in vivo, disclose the possibility that CFTR Ser511 can be phosphorylated by the combined action of tyrosine kinases and CK2 and disclose a new mechanism of hierarchical phosphorylation where the role of the priming kinase is that of removing negative determinant(s).

Keywords CK2 · Lyn · CFTR · Hierarchical phosphorylation · Ser511 · NBD1

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Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is an ABC family membrane protein of 1,480 amino acids functioning as an anion channel. Cystic fibrosis is caused by mutations of the CFTR gene: although there are more than 1,900 documented mutations of CFTR (http://www.genet.sickkids.on.ca), the by far commonest one, which is found in about 70 % of CF causing alleles, is the deletion of phenylalanine 508 (F508del) located in the first nucleotide-binding domain (NBD1) (Lubamba et al. 2012). The F508del mutation by itself neither abrogates CFTR activity as a chloride channel (Dalemans et al. 1991) nor affects ATP binding to NBD1, whose structure is indeed almost identical to that of wild type NBD1 (Atwell et al. 2010). The F508del mutant, however, fails to traffic to the plasma membrane, being retained in the ER by the cellular protein quality control machinery, retro-translocated from ER and proteolytically degraded via the proteasome (Määttänen et al. 2010). Consequently <1 % of F508delCFTR reaches the membrane (when compared with >50 % CFTR wild type) (Riordan 2008) and even the small amount that reaches the membrane is less stable than its wild type counterpart as judged from its much shorter half life (Heda et al. 2001).

As a high resolution structure of the complete CFTR molecule is not available, the precise folding defect caused by the F508 deletion remains a matter of conjecture. It is known that the F508del mutation although having no effect on the structure of the isolated NBD1 domain, nevertheless destabilizes NBD1 by disrupting its interactions with the cytoplasmic loop 4 (CL4) in the second MSD (Thibodeau et al. 2010; Serohijos et al. 2008; Mendoza et al. 2012; Rabeh et al. 2012). These interactions involve also other residues flanking F508 (Hunt et al. 2013), a circumstance that makes

particularly intriguing the presence close to the C terminal side of F508 of a seryl residue (Ser511) fulfilling the consensus sequence for being phosphorylated by protein kinase CK2 (S/T-x-x-E/D). Interestingly, this CK2 motif is highly conserved, suggesting a functional role for it; however, despite several efforts, it looks refractory to CK2 phosphorylation (Pagano et al. 2008): in fact neither the full size NBD1 domain, where Ser511 is well exposed, nor peptides reproducing the amino acid sequence encompassing it are phosphorylated by CK2, regardless to the presence or not of F508 in them. This prompted us to perform a study aimed at understanding why Ser511 is not susceptible to phosphorylation by CK2 despite it displays the right consensus and to assess if such a hindrance can be overcome by physiological mechanism(s) that may help to explain the instability of F508delCFTR. The outcome of this study is presented in this report.

Materials and methods

Materials

[γ^{33}P]ATP was purchased from Perkin-Elmer (Waltham, MA), PP2 (4-amino-5(4chlorophenyl)7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine) from Calbiochem (Darmstadt, Germany) and other chemicals from Sigma-Aldrich (Dorset, UK). Murine NBD1 domains, wild type and F508del, spanning sequence 389–673, were generously provided by the Philip J. Thomas Laboratory (Southwestern Medical Center, University of Texas, Dallas TX, USA).

Enzymes

Tyrosine kinases Lyn, Fgr, and Syk were purchased from Millipore Corporation (Billerica, MA). Human recombinant CK2 holoenzyme ($\alpha_2\beta_2$) was prepared essentially as described in Sarno et al. (1996) with some modifications. Briefly, human CK2α and CK2β were expressed in *Escherichia coli* independently. CK2$\alpha_2\beta_2$ holoenzyme was reconstituted by mixing pellets before mechanical lysis in Tris/HCl 25 mM, pH 8, NaCl 0.4 M, DTT 1 mM, and proteases inhibitors cocktail (Roche, Mannheim, Germany). The supernatant obtained by a 30 min centrifugation at 15,000*g* was then loaded on Heparin-Sepharose and the proteins were separated using a gradient elution (0.4–1 M NaCl). Fractions containing CK2 were further purified by size-exclusion chromatography using a buffer composed by Tris/HCl 25 mM, pH 8, NaCl 0.5 M, DTT 1 mM.

Peptide synthesis

Synthetic CFTR peptides were prepared by solid phase peptide synthesis method using a multiple peptide synthesizer (SyroII, MultiSynTech GmbH) on 4-hydroxymethylphenoxyacetyl PEGA resin (Novabiochem, Bad Soden, Germany). The fluoren-9-ylmethoxycarbonyl (Fmoc) strategy (Fields and Noble 1990) was used throughout the peptide chain assembly, utilizing *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) as coupling reagent (Carpino et al. 2001). The side-chain protected amino acid building blocks used were: *N*-α-fmoc-Nω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine, *N*-α-fmoc-γ-*tert*-butyl-L-glutamic acid, *N*-α-fmoc-β-*tert*-butyl-L-aspartic acid, *N*-α-fmoc-*O*-*tert*-butyl-L-tyrosine, *N*-α-fmoc-*O*-*tert*-butyl-L-serine, *N*-α-fmoc-*O*-benzyl-phospho-L-serine, *N*-α-fmoc-O-*tert*-butyl-L-tyrosine, *N*-α-fmoc-*O*-benzyl-phospho-L-tyrosine, *N*-α-fmoc-*N*ε-(*tert*-butyloxycarbonyl)-L-lysine, *N*-α-fmoc-*N*(im)-trityl-L-histidine, *N*-α-fmoc-*N*-γ-trityl-L-glutamine, *N*-α-fmoc-*S*-trityl-cystine, and *N*-α-fmoc-*N*-β-trityl-L-asparagine. Cleavage of the peptide was performed by reacting the peptidyl-resins with a mixture containing TFA/ethanedithiol/phenol 5 % for 2.5 h. Crude peptide was purified by a preparative reverse phase HPLC. Molecular masses of the peptide were confirmed by mass spectroscopy on a MALDI TOF–TOF mass spectrometer [model 4800, Applied Biosystems (Carlsbad, CA)]. The purity of the peptides was in the range 80–90 % as evaluated by analytical reverse phase HPLC.

Phosphorylation assays

Lyn, Fgr, and Syk activities were assayed in a final volume of 25 µl containing 50 mM Tris/HCl (pH 7.5), 10 mM $MnCl_2$, 20 µM [γ-^{33}P]ATP (about 1,000 c.p.m./pmol), 10 mU of enzymes and the indicated amounts of substrates. In the case of CK2, 12 mM $MgCl_2$ was used instead of $MnCl_2$ and 100 mM NaCl was added to the phosphorylation medium. After 10 min at 30 °C, samples containing NBD1 domains were subjected to SDS-PAGE (11 %) and protein phosphorylation was analyzed by a Cyclone Storage Phosphor-Screen (PerkinElmer). Samples containing peptides with an Arg_3 tag [CFTR(508–515)] were processed by phospho-cellulose paper procedure (Glass et al. 1978) and peptide phosphorylation was measured in a Scintillation Counter. A control devoid of peptide substrates was run in parallel and subtracted from the assays where peptides were present. Longer peptides [CFTR(500–523)] were separated from [γ-^{33}P]ATP by tricine-SDS-PAGE (Schagger 2006) and their phosphorylation was quantified by a Cyclone Storage Phosphor-Screen (PerkinElmer).

Kinetic determination

The Michaelis–Menten kinetic constants for peptide phosphorylation were calculated by nonlinear regression using GraphPad Prism software (La Jolla, CA).

Fig. 1 Conserved CK2 consensus close to F508 in CFTR. The potential CK2 target (Ser511) and its crucial acidic determinant at position $n + 3$ (Glu514) are *bold typed* and *underlined*. The additional positive determinant at position $n + 2$ (Asp513) is also *underlined*. Phe508, whose deletion is the commonest cause of cystic fibrosis, is *bold typed* in *italics*. Gly509 and Val510 play a critical role in the local perturbation caused by Phe508 deletion. An asterisk denotes residues, which have never been found mutated in the course of CFTR genetic screenings. Additional details are given in the text

Results

Ser511 paradox

A potential phospho-acceptor site for protein kinase CK2 (Ser511) is present in the nucleotide-binding domain 1 (NBD1) of CFTR, close to the phenylalanine residue (F508), whose deletion is the commonest cause of cystic fibrosis (F508del). As shown in Fig. 1, not only this site fulfills the CK2 consensus (S/T-x-x-E/D), but it also includes an additional acidic determinant at position $n + 2$ which is expected to further improve its susceptibility to phosphorylation (Pinna and Ruzzene 1996; Meggio and Pinna 2003). This site moreover is remarkably conserved with only one case of Ser511 mutation (to Cys) having been reported in the course of countless CFTR genetic screens performed worldwide (http://www.genet.sickkids.on.ca); no mutations of Tyr512 and Glu514 at the crucial positions ($n + 1$/$n + 3$) for substrate recognition by CK2, have been found to date and the same applies to the two residues adjacent to the N-terminal side of Ser511, Val510, and Gly509, both crucially involved in the local alterations caused by the F508 deletion (Hunt et al. 2013). Intriguingly, however, Ser511 appears to be entirely refractory to phosphorylation by CK2 either using the whole NBD1 domain or shorter peptides as phosphorylatable substrates, regardless to the presence or deletion of F508 (Pagano et al. 2008). This rises the question of why a potential phosphorylation site for CK2, highly conserved and exposed on the NBD1 surface is not susceptible to phosphorylation. Its proximity to the crucial F508 residue makes the question particularly tantalizing. A logical explanation could be that a functionally meaningful phosphorylation of Ser511 indeed takes place in living cells, but is normally prevented by local negative determinant(s) which can be removed under particular circumstances.

Tyr512 operates as a negative determinant preventing Ser511 phosphorylation by CK2

In an attempt to disclose local negative determinants preventing the phosphorylation of Ser511 by CK2 a series of short peptides was generated, in which residues flanking Ser511 were individually replaced by alanine. As shown in Table 1 neither the parent peptide nor any of the substituted peptides were significantly phosphorylated by CK2, with the notable exception of the peptide in which Tyr512 was replaced by alanine (peptide 4). This peptide is readily phosphorylated by CK2 indicating that the presence of Tyr512 in the natural sequence is responsible for the failure of CK2 to phosphorylate Ser511 although this residue displays the proper consensus for being phosphorylated. To note that phosphorylation is not restored if Tyr512 is replaced by Phe instead of Ala (peptide 8), while it is dramatically enhanced if Tyr512 is replaced by aspartic acid (peptide 9). Both outcomes were in some way expectable considering on one side that Phe is negatively selected at position $n + 1$ in CK2 sites (Hornbeck et al. 2011), on the other that, in contrast, an aspartic acid at position $n + 1$ is perceived as a powerful positive determinant by CK2 (Meggio and Pinna 2003; Salvi et al. 2009), being indeed the most positively selected residue in CK2 sites (Hornbeck et al. 2011). Considering on the other hand that phospho-tyrosine has been also shown to act as a positive determinant of CK2-mediated phosphorylation (Meggio et al. 1991), the results of Table 1 suggest that phosphorylation of Tyr512 could represent a physiological device to "unlock" the CK2 site, rendering Ser511 susceptible to phosphorylation. Indeed the in vitro phosphorylation of Tyr512 by protein tyrosine kinase Syk has been reported (Mendes et al. 2011; Luz et al. 2011) and another tyrosine just down-stream from

Table 1 Susceptibility of variably substituted peptides encompassing CFTR Ser511 to CK2 phosphorylation

		Phosphorylation rate (cpm min^{-1})
1	RRRFGV<u>S</u>YDEY[a]	n.d.[b]
2	RRR**A**GV<u>S</u>YDEY	n.d.
3	RRRFG**A**<u>S</u>YDEY	n.d.
4	RRRFGV<u>S</u>**A**DEY	2,287
5	RRRFGV<u>S</u>Y**A**EY	n.d.
6	RRRFGV<u>S</u>YD**A**Y	n.d.
7	RRRFGV<u>S</u>YDE**A**	n.d.
8	RRRFGV<u>S</u>**F**DEY	n.d.
9	RRRFGV<u>S</u>**D**DEY	18,016

Ser511 is underlined. Substituted residues are bold typed. Peptides (100 μM) were phosphorylated and processed as detailed in "Materials and methods". Peptide phosphorylation is expressed as cpm min^{-1}. Data are means of three separate experiments. SEM values were always <16 %

[a] The parent peptide (1) reproduces the 508–515 CFTR sequence with the N-terminal addition of an arginyl triplet to make applicable the phospho-cellulose paper method

[b] *n.d.* not detectable (<250 cpm min^{-1})

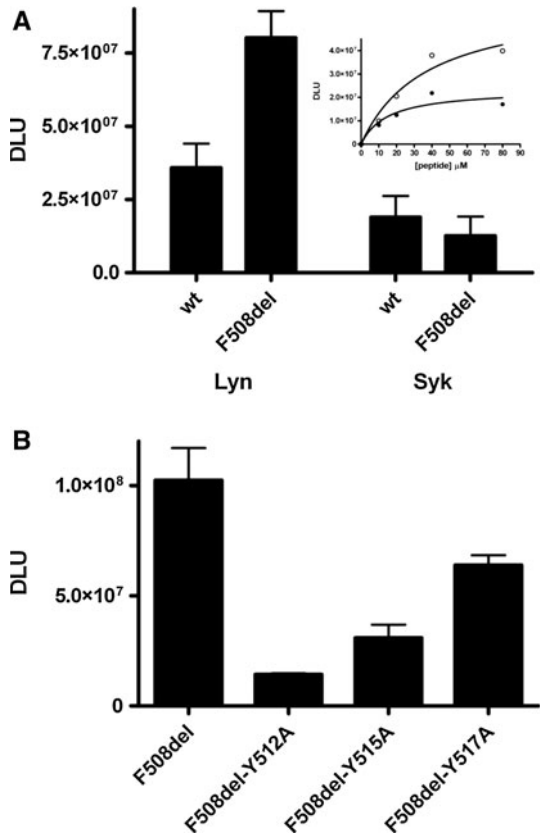

Fig. 2 Peptides reproducing the 500–523 sequence of CFTR are readily phosphorylated by the Src family tyrosine kinase Lyn in a manner which is increased by the F508 deletion. The sequence of the wild type peptide was the following: ^{500}GTIKENII**F**^{508}GVSYDEYRYRSVIKA^{523}. F508 (*bold typed*) was lacking in the F508del peptide, in whose Tyr substituted derivatives Y512, Y515 and Y517 (*underlined*) were individually replaced by alanine. **a** Wild type (wt) and F508del peptides (80 μM) were phosphorylated by Lyn and Syk as detailed in "Materials and methods". **b** F508del peptide and its derivatives with tyrosyl residues individually replaced by alanine were phosphorylated by Lyn. Samples were subjected to tricine-SDS-PAGE and peptide ^{33}P-phosphorylation, quantified by a Cyclone Storage Phosphor-Screen, is expressed as Digital Light Units (DLU)

Tyr512, Tyr515 is among CFTR residues which are phosphorylated in vivo (Wang et al. 2006).

Phosphorylation of Tyr512 triggers subsequent phosphorylation of Ser511 by CK2

To gain information about the susceptibility to phosphorylation of tyrosyl residues downstream from CFTR Ser511, a peptide encompassing residues 500–523 of CFTR, either wild type or with the F508 deletion (F508del), was synthesized and subjected to phosphorylation by protein tyrosine kinases of the Src and Syk families. As shown in Fig. 2a, the wild type peptide was readily phosphorylated by the Src family kinase Lyn, and to a lesser extent by Syk. Phosphorylation by Fgr, another member of the Src family, could be also observed (not shown). Interestingly,

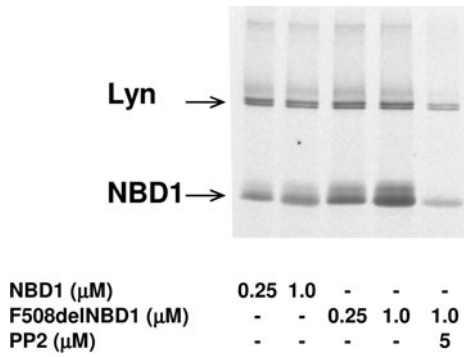

Fig. 3 F508delNBD1 is phosphorylated by Lyn more readily than wild type NBD1. The indicated amounts of wild type and F508del-NBD1 domains were phosphorylated by Lyn, in the absence or presence of the Lyn inhibitor PP2, and subjected to SDS-PAGE. Protein ^{33}P-phosphorylation was analyzed in a Cyclone Storage Phosphor-Screen. Upper band refers to ^{33}P-autophosphorylated Lyn. The figure is representative of three separate experiments

phosphorylation by Lyn (but not by Syk) was markedly enhanced by the deletion of F508. For the F508del peptide, a Km around 37 μM was calculated for Lyn phosphorylation. An even lower value (15 μM) was calculated for the phosphorylation of the wild type peptide, whose phosphorylation rate, however, is much lower (inset of Fig. 2a). Preferential phosphorylation by Lyn of the F508del substrate was confirmed using the full size NBD1 (Fig. 3): as in the case of the peptide, F508delNBD1 was phosphorylated more readily than its wild type counterpart.

To assess which tyrosyl residue(s) are phosphorylated by Lyn, derivatives of the F508delCFTR 500–523 peptide with the three tyrosines individually replaced by alanine were synthesized and assayed for their phosphorylation. As shown in Fig. 2b, substitution of Tyr512 almost entirely suppressed phosphorylation, whereas substitution of Tyr515 and even more of Tyr517 was less detrimental. These data support the view that the main target of Lyn is Tyr512, with Tyr515 and, to a lesser extent Tyr517, being also required for optimal phosphorylation.

Next step was therefore to synthesize a F508delCFTR 500–523 phospho-peptide with phospho-tyrosine replaced for Tyr512 and to compare its phosphorylation with those of the peptides with either tyrosine or alanine at position 512. The results are reported in Fig. 4 and they show that, as already observed with shorter peptides (see Table 1), the parent peptide is not affected by CK2, whereas the Ala substituted one is readily phosphorylated. More remarkably however the phospho-tyrosyl peptide is the best substrate, providing the incontrovertible evidence that phosphorylation of Tyr512 makes Ser511 a good target for CK2.

In an attempt to see if such a kind of hierarchical phosphorylation also takes place within the whole NBD1 domain, whose three-dimensional structure is unchanged when it participates to the whole CFTR protein, the F508delNBD1

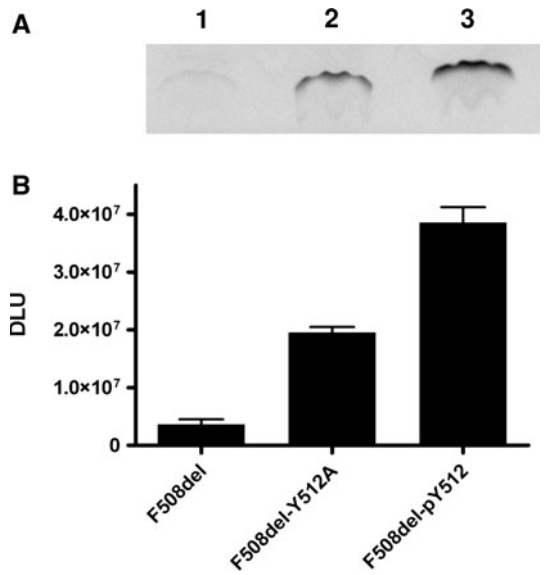

Fig. 4 Previous phosphorylation of Tyr512 makes Ser511 susceptible to phosphorylation by CK2. Peptides reproducing the 500–523 sequence of F508delCFTR were synthesized either as such (see legend of Fig. 2) or with Tyr512 replaced by Ala (Y512A) or by phospho-tyrosine (pY512). Peptides (60 μM) were incubated with [γ^{33}P]ATP and CK2, and subjected to tricine-SDS-PAGE as detailed in "Materials and methods". ^{33}P incorporated into the peptides was analyzed (**a**) and quantified (**b**) by a Cyclone Storage Phosphor-Screen. Lanes *1*, *2* and *3* refer to unsubstituted, Y512A and pY512 peptides, respectively. Means of DLU values relative to three separate experiments are reported in histograms (**b**)

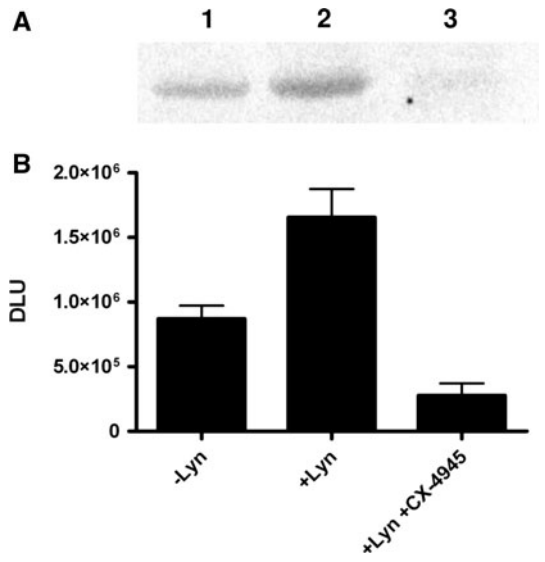

Fig. 5 Preincubation with ATP-Mn^{++} and Lyn makes F508del-NBD1 more susceptible to subsequent CK2-mediated phosphorylation. F508delNBD1 domain (1 μM) was incubated in a phosphorylation medium containing 20 μM of unlabeled ATP in the absence (lane *1*) or presence (lanes *2*, *3*) of Lyn tyrosine kinase as described in "Materials and methods". After 30 min, the Lyn inhibitor PP2 (10 μM) was added in all samples. [γ^{33}P]ATP (1,000 c.p.m./pmol) was then added together with recombinant human CK2 catalytic subunit under conditions described elsewhere for NBD1 phosphorylation by CK2 (Pagano et al. 2008), either in the absence (lanes *1*,*2*) or presence (lane *3*) of the CK2 inhibitor CX-4945 (5 μM) for 15 min. Samples were subjected to SDS-PAGE and the domain ^{33}P-phosphorylation was analyzed (**a**) and quantified (**b**) by a Cyclone Storage Phosphor-Screen. Bar graph (**b**) reports the means of DLU values relative to three separate experiments

domain was subjected to phosphorylation by ^{33}P-ATP in the presence of CK2 and the Lyn inhibitor PP2, after 30 min incubation either in the presence or not of Lyn and unlabeled ATP. The interpretation of this experiment may be biased by the fact that, besides Ser511 and Tyr512, additional phospho-acceptor sites for both CK2 and Lyn are present in NBD1 (Pagano et al. 2008, and this paper), not to say about the difficulty to attain significant phosphorylation stoichiometry by incubation with tyrosine kinases. With all these caveats it was possible to conclude from the data shown in Fig. 5 that indeed a hierarchical effect is observable for CK2-mediated radiolabeling upon previous phosphorylation by Lyn. In fact the radiolabeling of F508delNBD1 is significantly increased upon preincubation with Lyn, such an increment being drastically reduced by addition of the very specific CK2 inhibitor CX-4945. In parallel experiments with radiolabeled ATP, the amount of phosphate incorporated by Lyn was determined and found to correspond to a stoichiometry approaching 0.13 mol phosphate. mol protein^{-1} (not shown).

Discussion

The data presented in this report on one side provide an explanation to a vexed question related to inability of CFTR Ser511 to be phosphorylated by protein kinase CK2 despite it displays the canonical consensus sequence (S-x-x-E) for undergoing phosphorylation, on the other disclose the possibility that the biological properties of CFTR might be regulated by the combined action of tyrosine kinase(s) and CK2.

As far as the first issue is concerned by adopting an Ala scan substitution approach, we show that failure of CK2 to phosphorylate Ser511 is primarily due to a local negative determinant, namely the tyrosyl residue adjacent to the C terminal side of Ser511 (Tyr512): its replacement by Ala in fact is sufficient to "unlock" the phospho-acceptor site, rendering it susceptible to CK2 catalyzed phosphorylation. In contrast Ala substitutions at different positions surrounding Ser511 fail to promote its phosphorylation by CK2. The detrimental effect of Tyr512 seems to be due to its bulky aromatic side chain since its replacement with Phe, unlike Ala and Asp, does not restore phosphorylation of Ser511. Indeed "two-sample logo" analyses of bona fide CK2 sites reveal that Phe, similar to other bulky hydrophobic side chains, is negatively selected at position $n + 1$ (Hornbeck et al. 2011), suggesting that it plays a negative role as specificity determinant.

It should be noted that in the case of CFTR Ser511 both the "negative" determinant, Tyr512 and the positive one, Glu514 are highly conserved (Fig. 1), suggesting that evolution led to a deliberately "blocked" CK2 site. This in turn would imply that such a blockage should be reversible under specific biological conditions. We reasoned that the only physiological manner to overcome the negative effect of Tyr512 would be a naturally occurring post-translational modification of its side chain, with special reference to its phosphorylation. The occurrence of such a mechanism was supported by a report showing in cells the phosphorylation of Tyr512 (Mendes et al. 2011).

The mechanism of Ser511 phosphorylation outlined here can also provide an explanation for the finding that two CFTR mutants, in which Tyr512 has been replaced by Ala and Asp, respectively, undergo defective maturation with reduced cell surface exposition (Luz et al. 2011). Since we have shown here that both these mutations make Ser511 susceptible to CK2 phosphorylation, impaired maturation could be accounted for by phosphorylation of Ser511 rather than by the Tyr512 mutation per se. Consistent with this interpretation it has been recently found that by treating F508delCFTR expressing cells with the highly specific CK2 inhibitor CX-4945, newly synthesized F508delCFTR accumulates more than in untreated cells (Venerando et al. 2013). Pertinent to this may be the observation that in our hands the F508delCFTR peptide is phosphorylated by Lyn more readily than the wild type one (Fig. 2a) and the same applies to the whole NBD1 domain (Fig. 3), suggesting that the sequential phosphorylation mechanism outlined by our work mainly affects the F508del mutant and could be somehow instrumental to the detrimental effect of the F508 deletion on CFTR stability. It may be also worthy to note in this respect that our peptide is phosphorylated by the Src kinases Lyn and Fgr more readily than it is by Syk. Moreover, Lyn also phosphorylates Tyr515 (Fig. 2b), a residue that was found to undergo phosphorylation in living cells (Wang et al. 2006), but which is not affected by Syk (Luz et al. 2011). Taken together, these observations in conjunction with a recent report showing a role of the Src tyrosine kinase family in the muscarinic activation of CFTR (Billet et al. 2013) support the view that Lyn or other PTKs with similar specificity are the natural agents that phosphorylate CFTR downstream from Ser511. On the other hand, failure to come across phosphoTyr512 (as well as phosphoSer511) in the repertoires of human phospho-peptides available to date may be not surprising if it is considered on one side that such a phosphorylation seems to affect preferentially the F508del mutant (Figs. 2, 3), which is present only in individuals bearing this mutation, on the other the possibility that F508delCFTR, once phosphorylated at Ser511, is rapidly degraded. Indeed several examples are known of proteins which are committed to proteolytic degradation by CK2-catalyzed phosphorylation (Torres and Pulido 2001; Kato et al. 2003; Scaglioni et al. 2006).

While additional work will be needed to identify the Tyr kinase(s) responsible for CFTR phosphorylation at Tyr 512/515 and to validate in vivo the occurrence of Ser511 phosphorylation by the combined intervention of CK2 and tyrosine kinase(s), this mechanism deserves a comment because it represents a special case of "hierarchical phosphorylation" (Roach 1991; Pinna and Ruzzene 1996). This latter would imply the generation of the consensus sequence for a "phosphate directed kinase" by a "priming" kinase, while in the case of CFTR Ser511 the consensus sequence (S^{511}-y-d-E^{514}) is already there but is not viable due to Tyr512 playing the role of "negative determinant". The tyrosine kinase, in this case, rather than "priming" phosphorylation by CK2, removes such a negative determinant and restores the efficacy of the pre-existing consensus. This is conceptually noticeable as it adds a new facet, to the best of our knowledge, to the complexity of events that cooperate to determine the selectivity of protein phosphorylation (Ubersax and Ferrel 2007).

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Conflict of interest The authors declare that they have no conflict of interest.

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