Modulation of Protein Kinase CK2 Activity by Fragments of CFTR Encompassing F508 May Reflect Functional Links with Cystic Fibrosis Pathogenesis†

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ABSTRACT: Deletion of F508 in the first nucleotide binding domain (NBD1) of cystic fibrosis transmembrane conductance regulator protein (CFTR) is the commonest cause of cystic fibrosis (CF). Functional interactions between CFTR and CK2, a highly pleiotropic protein kinase, have been recently described which are perturbed by the F508 deletion. Here we show that both NBD1 wild type and NBD1 ∆F508 are phosphorylated in vitro by CK2 catalytic α-subunit but not by CK2 holoenzyme unless polylysine is added. MS analysis reveals that, in both NBD1 wild type and ∆F508, the phosphorylated residues are S422 and S670, while phosphorylation of S511 could not be detected. Accordingly, peptides encompassing the 500–518 sequence of CFTR are not phosphorylated by CK2; rather they inhibit CK2α catalytic activity in a manner which is not competitive with respect to the specific CK2 peptide substrate. In contrast, 500–518 peptides promote the phosphorylation of NBD1 by CK2 holoenzyme overcoming inhibition by the β-subunit. Such a stimulatory efficacy of the CFTR 500–518 peptide is dramatically enhanced by deletion of F508 and is abolished by deletion of the I507 doublet. Kinetics of NBD1 phosphorylation by CK2 holoenzyme, but not by CK2α, display a sigmoid shape denoting a positive cooperativity which is dramatically enhanced by the addition of the ∆F508 CFTR peptide. SPR analysis shows that NBD1 ∆F508 interacts more tightly than NBD1 wt with the α-subunit of CK2 and that CFTR peptides which are able to trigger NBD1 phosphorylation by CK2 holoenzyme also perturb the interaction between the α- and the β-subunits of CK2.

By far, the most common cause of cystic fibrosis is the deletion of a single amino acid, phenylalanine 508 (∆F508), in the nucleotide binding domain-1 (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is an ion channel belonging to the ATP-binding cassette (ABC) family of transmembrane pumps, but unlike other family members, CFTR displays no known pump activity (1). Protein phosphorylation and adequate nucleotide levels play a key role in the control of CFTR channel function, particularly activation by PKA, augmentation by PKC (2), and inhibition by AMPK (3, 4), but their interactions are complex and incompletely understood (5). This complexity arises in part from many observations suggesting that CFTR is part of a multimolecular complex in the apical membrane of epithelial cells containing (besides protein kinases) N-terminal inhibitory syntaxins, PKA-interacting ezrin binding phosphoprotein (6), and many others including CAP 70 (7) and more recently a cAMP-efflux pump binding at the C-terminus of CFTR (8). When CFTR is purified to homogeneity, F508 deletion by itself, albeit causing a significant gating defect (9), neither prevents CFTR activity as chloride channel (10) nor affects ATP binding by NBD1, whose overall structure is unlikely to critically rely on F508 because this residue is located in a flexible loop on the periphery of the overall structure remote from the ATP binding site (11). Nevertheless, this mutation leads to reduced CFTR channel function with current models suggesting poor retention in the plasma membrane after loss of F508 (12). This may result from improper folding (13) and instability of CFTR whose susceptibility to the protein degradation machineries is therefore increased (14, 15). One school of thought suggests that less than 1% of the ∆F508 CFTR reaches the membrane, where it can display some attenuated activity (9), while this figure in the case of wild-type CFTR can approach 75% in some cell types and culture conditions (16). There are some dissenting views (15) and even the well established idea that CFTR devoid of F508 fails to fold has recently been challenged (17).

A possible alternative explanation for the dramatic effects of F508 deletion could be that this residue is directly or indirectly implicated in interactions between CFTR and the

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* Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator protein; NBD, nucleotide binding domain; CK2, casein kinase-2.
network of proteins committed, on the one hand, to its proper folding and processing and, on the other, to its unfolding and degradation. Alternatively, F508 might be important for the interaction with a regulatory protein given its accessible location in NBD1. The latter idea might provide a means to explain the multisystem nature of cystic fibrosis provided the regulatory protein has multiple targets. Pertinent to this location in NBD1. The latter idea might provide a means to folding and processing and, on the other, to its unfolding network of proteins committed, on the one hand, to its proper subunits (implicated in protein synthesis, folding, and degradation) by an acidic side chain at position kinase which recognizes seryl and threonyl residues specified in protein kinase CK2. CK2 is a highly pleiotropic protein residue, S511, located within a consensus sequence for the kinase CK2 were expressed in Escherichia coli and purified as previously described (25). Native CK2 was purified from rat liver (26).

Peptide Synthesis. The CFTR-derived synthetic peptides were prepared by the solid-phase peptide synthesis method using an automatized peptide synthesizer (model 431-A; Applied Biosystems, Foster City, CA) as C-terminal acids on HMP resin (Applied Biosystems) or as C-terminal amides on Rink Amide PEGA resin (Novabiochem, Bad Soden, Germany). The fluoren-9-ylmethoxycarbonyl (Fmoc) strategy (27) was used throughout the peptide chain assembly. The No-Fmoc amino acids carrying standard side chain protective groups were converted to benzotrizoiyl esters with 1-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). N-Terminal acetylation was performed on the peptidyl-resin using acetic anhydride. Cleavage of the peptides was performed by reacting the peptidyl resins with a mixture containing TFA/H2O/thioanisole/ethanediol/phenol (10 mL/0.5 mL/0.5 mL/0.25 mL/750 mg) for 2.5 h. Crude peptides were purified by a preparative reverse-phase HPLC. Molecular masses of the peptides were confirmed by mass spectroscopy with direct infusion on a Micromass ZMD-4000 mass spectrometer (Waters-Micromass). The purity of the peptides was about 95% as evaluated by analytical reverse-phase HPLC.

Phosphorylation Assay. In vitro phosphorylation of NBD1 proteins and of the NBD1-derived synthetic peptides was performed by incubating substrates (final volume 25 µL) in a medium containing 50 mM Tris-HCl, pH 7.5, 12 mM MgCl2, 100 mM NaCl, and 100 µM [γ-33P]ATP (specific radioactivity 2000–4000 cpm/pmol) in the presence of CK2α catalytic subunit (8–28 nM) or CK2 holoenzyme (0.8–4.8 nM). In some experiments polylysine (330 nM) was added. The concentration of NBD1 was 3.5 µM unless differently specified in figure legends. The reaction was stopped by addition of Laemmli buffer and subjected to SDS–PAGE. Protein samples were visualized by staining with Coomassie Brilliant Blue or, alternatively, transferred onto nitrocellulose membranes and stained with Ponceau red dye. Dried gels or membranes were then exposed to storage phosphor screens overnight, which were subsequently scanned by the Cyclone Storage Phosphor System (Packard). After scanning was complete, a resulting digitized image could be viewed and analyzed by the Optiquant software, which expresses radioactivity as digital light units (DLU). Whenever phosphate incorporation into protein substrates was required to assess catalytic efficiency, the relationship between the DLU and 33P cpm was deduced by spotting known amounts of [γ-33P]ATP (specific activity 7000 Ci/mmol) in triplicates on filter paper, which were dried and exposed to the phosphor screen together with the sample. From the DLU values and

**EXPERIMENTAL PROCEDURES**

**Materials.** Polylysine (M, 47000) and most of the reagents were purchased from Sigma. Purified wild-type and ΔF508 mutated recombinant murine NBD1 (spanning sequence 389–673) and wild-type human NBD1 (spanning sequence 389–673) were generously provided by the Philip J. Thomas laboratory (Southwestern Medical Center, University of Texas, Dallas, TX; http://www4.utsouthwestern.edu/thomaslab/). Recombinant α- and β-subunits of human protein kinase CK2 were expressed in Escherichia coli and purified as previously described (25). Native CK2 was purified from rat liver (26).

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Solutions of the interacting proteins, NBD1 wt and NBD1
\( \Delta F508 \) (Thermo Electron, San Jose, CA) interfaced with
human wild-type CFTR nucleotide binding domain 1
and CK2
determine the kinetic constants of the interactions. CK2
phospho residue(s).

The phosphorylation of mouse wild-type, mouse
\( \Delta F508 \) mutated NBD1 by protein kinase CK2. In (A) 3 \( \mu \)g of murine and human NBD1 were incubated under conditions decribed in the Experimental Procedures with CK2\( \alpha \) catalytic subunit either alone (lane 1) or previously combined with equimolar amounts of CK2/\( \beta \) regulatory subunit in the absence (lane 2) or in the presence of 330 nM polylysine (lane 3). Only the autoradiograms are shown. In (B) and (C) the kinetics of phosphorylation by CK2 catalytic subunit and by CK2 holoenzyme, respectively, at increasing concentration of NBD1 wild type (\( \Delta F508 \)) and (\( \Delta F508 \)) are illustrated. The data represent the means obtained from experiments run in triplicate with SD never exceeding 15%.

**RESULTS**

The phosphorylation of mouse wild-type, mouse \( \Delta F508 \),
and human wild-type CFTR nucleotide binding domain 1
(ND1) by protein kinase CK2, either catalytic subunit or
reconstituted holoenzyme, is shown in Figure 1A: mouse
ND1 \( \Delta F \) is phosphorylated by CK2\( \alpha \) slightly better than
its wild-type counterpart which in turn is phosphorylated
more readily than the wild-type human homologue. Phos-
phorylation is abolished in all cases if the catalytic subunits
of CK2 are combined with the \( \beta \)-subunits to form CK2
holoenzyme. The latter is in fact inactive toward all NBD1
forms unless polylysine is present. Polylysine overcomes
downregulation by the \( \beta \)-subunit and stimulates NBD1 (wild
type and \( \Delta F508 \) phosphorylation by the holoenzyme beyond
the level observed with the catalytic subunit alone. Addition
of polylysine to the \( \alpha \)-subunit alone has no significant effect
(not shown). Kinetic analyses of murine wild-type vs
\( \Delta F508 \) NBD1 phosphorylation by CK2 \( \alpha \)-subunit show that they
display a similar behavior (Figure 1B). Kinetics performed
with CK2 holoenzyme plus polylysine (Figure 1C) are also
similar with either NBD1 wild type or ∆F508. Note however that the apparent \( K_m \) values in this case are higher than those calculated with CK2-R, especially due to a sigmoid shape of the first part of the saturation curves.

To identify the residues phosphorylated by CK2, NBD1 either wild type or ∆F508 were exhaustively phosphorylated by CK2 holoenzyme in the presence of polylysine and elution of the radiolabeled bands from the SDS–PAGE gel were performed as described in the Experimental Procedures. The position of the phospho residue is marked with an asterisk. Note the presence of several semitryptic peptides probably due to a contamination with chymotrypsin. (A) List of identified phosphopeptides. Experimental mass, charge state of the peptides, theoretical mass, and delta mass are listed with the peptide sequence. The position of the phospho residue is marked with an asterisk. Note the presence of several semitryptic peptides probably due to a contamination with chymotrypsin. (B) Annotated fragmentation spectra of the phosphopeptides HS*SDENNVSFSH (left panel) and HS*S*DENNVSFSH (right panel). For the monophosphorylated peptide the position of the phosphoserine is clearly indicated by the transition \( y_{10} - y_{11} \), while for the bisphosphorylated peptide are very clear the transitions \( y_{9} - y_{10} \) and \( y_{10} - y_{11} \) that indicate the positions of the two phosphoserines. Both spectra are dominated by the neutral losses of \( H_3PO_4 \) from the parent ion. The label \( \Delta \) indicates the fragments originated by the neutral loss of phosphate.

| Exp. Mass (Da) | Charge State | Theor. Mass (Da) | Δ Mass (Da) | Sequence |
|----------------|-------------|-----------------|------------|----------|
| 818.32         | 1           | 818.31          | 0.01       | RFS*VDD  |
| 712.22         | 2           | 1422.49         | -0.06      | KHS*S*DENNVSFH |
| 720.44         | 2           | 1438.51         | 0.35       | HS*SDENNVSFSH |
| 760.38         | 2           | 1518.48         | 0.26       | HS*SDENNVSFSH |
| 776.95         | 2           | 1551.60         | 0.29       | HS*SDENNVSFSH |
| 785.29         | 2           | 1567.59         | 0.97       | KHS*SDENNVSFSH |
| 834.46         | 2           | 1646.58         | 0.34       | KHS*S*DENNVSFH |
| 824.45         | 2           | 1646.58         | 0.32       | KHS*S*DENNVSFH |
| 840.65         | 2           | 1679.69         | -0.41      | KHS*SDENNVSFSH |
| 561.05         | 3           | 1679.69         | 0.42       | KHS*SDENNVSFSH |
| 880.59         | 2           | 1759.72         | -0.54      | KHS*S*DENNVSFH |
| 1331.61        | 2           | 2660.24         | 0.95       | KHS*SDENNVSFSHCLVGNPVLK |
| 914.81         | 3           | 2741.19         | 0.21       | KHS*SDENNVSFSHCLVGNPVLK |

FIGURE 2: MS analysis of NBD1 phosphorylation sites by protein kinase CK2. Phosphorylation of mouse wild-type NBD1 by CK2 holoenzyme in the presence of polylysine and elution of the radiolabeled bands from the SDS–PAGE gel were performed as described in the Experimental Procedures. (A) List of identified phosphopeptides. Experimental mass, charge state of the peptides, theoretical mass, and delta mass are listed with the peptide sequence. The position of the phospho residue is marked with an asterisk. Note the presence of several semitryptic peptides probably due to a contamination with chymotrypsin. (B) Annotated fragmentation spectra of the phosphopeptides HS*SDENNVSFSH (left panel) and HS*S*DENNVSFSH (right panel). For the monophosphorylated peptide the position of the phosphoserine is clearly indicated by the transition \( y_{10} - y_{11} \), while for the bisphosphorylated peptide are very clear the transitions \( y_{9} - y_{10} \) and \( y_{10} - y_{11} \) that indicate the positions of the two phosphoserines. Both spectra are dominated by the neutral losses of \( H_3PO_4 \) from the parent ion. The label \( \Delta \) indicates the fragments originated by the neutral loss of phosphate.

that no peptides including pS423 alone could be found, suggesting that the phosphorylation of S423 is primed or at least facilitated by previous phosphorylation of S422. Indeed, seryl residues flanked by a phosphorylated and a carboxylic side chain are known to become susceptible to CK2 (30). In human NBD1 S422 is conserved but S423 is not, and the sequence around S670 is altered. This may account for its reduced phosphorylation as compared to mouse NBD1 (see Figure 1A). S422 was also found to be the main or the only phosphorylated residue whenever NBD1 wild type and ∆F508 were phosphorylated by CK2 holoenzyme activated by stimulatory peptides (see below) instead of polylysine (see Figure 2). A remarkable and recurrent outcome of our MS analyses was the failure to detect any trace of phospho-
Correlation S511, conforming to the CK2 consensus sequence (S511YDE), despite the proposal that this was the first candidate for phosphorylation by CK2 (24). Lack of phosphorylated S511 was also consistent with failure of synthetic peptides encompassing the 500–518 CFTR sequence (listed in Figure 4) to undergo appreciable phosphorylation by either CK2R or CK2 holoenzyme plus polylysine. Neither the deletions of six and nine N-terminal residues nor substitution in the deleted 509–518 peptide of two downstream arginines (R516, R518), in the attempt to remove potentially negative determinants, was able to promote any significant phosphorylation of S511 peptides by CK2. A weak phosphorylation, negligible compared to full-length NBD1, however, was observed upon substitution of Tyr-512, a potential target for the Syk tyrosine kinase (31) with phosphotyrosine (peptide 5 in Figure 4) (not shown).

A possible explanation for the failure of the 500–518 peptide to undergo appreciable phosphorylation by CK2 despite the presence in it of the proper consensus sequence was that its phospho product is not easily released from the kinase, a circumstance frequently observed with peptide substrates of protein kinases (ref 32 and references therein). Such behavior reflects in the aptitude of such peptides to act as competitive inhibitors. We therefore examined the potential of the CFTR 500–518 peptide to inhibit the phosphorylation of NBD1 full length by CK2α. We found that, although at high peptide concentration (400 µM) the expected inhibition could be observed, at lower concentrations the peptide rather displayed a slight stimulatory effect which was more significant if the peptide had the F508 deletion (Figure 3A). This stimulatory effect was much more remarkable if CK2R was replaced by the holoenzyme, given that the latter alone (i.e., no polylysine present, Figure 1) is unable to phosphorylate NBD1 by itself. In this case, therefore, the peptide promotes a de novo phosphorylation of NBD1 which otherwise would be undetectable (Figure 3B), thus partially mimicking polylysine in this respect (see Figure 1). Again the ΔF508 peptide was more effective than the wild type in terms of both higher efficacy at lower concentration and overall phosphorylation increment. Note that in the case of the holoenzyme-mediated phosphorylation no inhibition could be observed even by increasing the concentration of the peptides to 800 µM. At such high concentration the stimulatory efficacy of the ΔF508 peptide and, to a lesser extent, of the wt peptide markedly declined. Similar data were obtained if the recombinant holoenzyme

![Figure 3](image-url) **Figure 3:** Dose-dependent effect of NBD1-derived synthetic peptides on the phosphorylation of NBD1 by protein kinase CK2. Phosphorylation of wild-type murine NBD1 (3 µg) was performed by CK2 catalytic subunit (panel A) and by CK2 holoenzyme (panel B) as described in the Experimental Procedures in the presence of increasing concentration of the NBD1-derived wild-type GTIKENIIGVSDEYR (C) and ΔF508 mutated GTIKENIIGVSYDEYR (O) peptides. The insets show the corresponding autoradiograms. The arrow indicates the position of NBD1. The data represent the means obtained from experiments run in triplicate with SD never exceeding 15%.

![Figure 4](image-url) **Figure 4:** Effect of variable substitutions within the NBD1-derived peptides on their stimulatory efficacy on NBD1 phosphorylation by CK2 holoenzyme. Murine NBD1 (3 µg) was phosphorylated by CK2 holoenzyme under conditions described in the Experimental Procedures in the presence of variably substituted peptides (160 µM) listed in (A). In (B) the SDS–PAGE corresponding autoradiograms are shown with arrows indicating the position of the NBD1 protein and of the autophosphorylated CK2β subunit, respectively. In (C) the quantitation of NBD1 radiolabeling with respect to the control in the absence of peptides (C) is reported as histograms. Numbering refers to the list reported in (A).
was replaced by native CK2 holoenzyme purified from rat liver (not shown).

In an attempt to gain insight into structural features responsible for the stimulatory efficacy of the CFTR 500–518 peptides, a number of derivatives bearing deletions and/or substitutions were synthesized and tested for their ability to promote the phosphorylation of NBD1 by CK2 holoenzyme. The data, summarized in Figure 4, show that the first six residues of the peptide GTIKENIFGSVDYED-RYR can be deleted without abrogating the stimulatory efficacy of the peptide. This stimulation is lost however if the first nine residues are deleted, thus disclosing the crucial role of the IIF508 triplet. On the other hand, the single deletion of F508 either in the parent peptide (500–518) or in its shortened derivative (506–518) enhances stimulation. A similar effect is observed if F508 is replaced by the smaller residue alanine, while its replacement with cysteine, a naturally occurring polymorphism devoid of the pathological consequences displayed by the ΔF508 mutation, has no effect. The crucial role of the 508 position is further highlighted by the finding that the stimulatory efficacy is decreased if F508 is replaced by the bulkier side chain of a tryptophan. On the other hand, the replacement of the potential CK2 target S511 by alanine (which does not occur naturally as S511 is conserved) significantly increases the stimulatory potency of the peptide. It may be pertinent to note that the efficacy of the peptides as stimulators of NBD1 phosphorylation correlates with their inhibition of CK2 autophosphorylation at its β-subunit (see lower bands in Figure 4B, for example), an event which is symptomatic of CK2 holoenzyme supramolecular organization (33). In contrast, no correlation with susceptibility to phosphorylation could be observed, given the inability of all the peptides listed in Figure 4A, with the only partial exception of the phosphopeptide 5 (devoid of any stimulatory efficacy) to undergo phosphorylation by CK2 (see also above).

Next we wanted to see if stimulation of CK2 holoenzyme by the CFTR 500–518 peptide and its ΔF508 derivative was a general property or was only evident with NBD1 and possibly other substrates whose phosphorylation is inhibited by the β-subunit (“class II” according to previous nomenclature (20)). To this purpose the efficacy of the peptides was evaluated using the following phosphoacceptor substrates: mouse NBD1, calmodulin, inhibitor-2 of protein phosphatase 1 (I-2), heat shock protein 90 (HSP90), and a specific peptide substrate of CK2 (R3AD2SD5). As shown in Figure 5A only in the case of NBD1 and calmodulin (the typical representative of class II substrates) was phosphorylation inhibited by the β-subunit, and in both cases it was restored by addition of the peptide. As in the case of NBD1, calmodulin phosphorylation is increased more efficiently by using the ΔF508 peptide instead of the wild-type CFTR peptide. In contrast, phosphorylation of I-2, HSP90, and the peptide substrate was not inhibited by the β-subunit, and no stimulation by the NBD1 wild-type peptide could be observed. However, a significant, near doubling, stimulation by the ΔF508 peptide was also evident with I-2 and HSP90 over and above that exerted in these cases by the β-subunit alone. It can be concluded therefore that only the phosphorylation of a subset of CK2 substrates whose phosphorylation is downregulated by the β-subunit alone is dramatically enhanced by the CFTR 500–518 segment acting differentially in an F508-dependent manner. The finding that the stimulatory effect of the β-subunit on the phosphorylation of the other substrates by CK2 holoenzyme is not decreased by the CFTR 500–518 wt peptide while being actually increased by the ΔF508 peptide would indicate that these peptides do not abrogate all the functions of the β-subunit, but only its downregulatory potential (compare the second and fourth bars for each protein substrate shown in Figure 5A).

Additional evidence supporting the crucial role of the β-subunit to mediate upregulation of CK2 activity by the CFTR-derived peptides came from experiments in which these peptides were tested for their ability to influence the phosphorylation of different substrates by CK2 α-subunit alone rather than by CK2 holoenzyme. Under these conditions as anticipated in Figure 3 NBD1 phosphorylation was affected in opposite ways depending on the peptide’s concentration, being stimulated at low concentration (around 100 μM) while inhibited at concentrations higher than 300 μM. In this respect, as shown in Figure 5B, only the phosphorylation of I-2 is reminiscent of NBD1 with a significant stimulation at 80 μM peptide and inhibition at 400 μM. With all of the other substrates the peptides were flatly inhibitory already at 80 μM. Concentrations below 80 μM were also tested and never found to display significant stimulatory efficacy (not shown). It has to be concluded therefore that the isolated catalytic subunit of CK2 generally is more susceptible to inhibition rather than to stimulation by the CFTR 500–518 peptides, the only exceptions being provided by the protein substrates NBD1 and I-2 at low peptide concentration. The drastic inhibition observed using the specific CK2 peptide substrate is especially remarkable considering that no inhibition at all is observed if the same experiment is run with CK2 holoenzyme (see Figure 5A). This suggests that inhibition is not merely due to pseudosubstrate effect. Accordingly, kinetics run with CFTR 500–518 peptides, either wild type or ΔF508 (Figure 5C), are consistent with a purely noncompetitive mechanism of inhibition. Collectively, our data strongly suggest that the allosteric site(s) on the α-subunit where the CFTR peptides bind is (are) no longer easily accessible in the holoenzyme.

Since the stimulatory efficacy of the CFTR peptides observed with CK2 holoenzyme is more pronounced if they bear the F508 deletion which is the most common cause of cystic fibrosis, it seems likely that these mutant peptides are better shaped to counteract the interactions with the β-subunit that are normally responsible for downregulation of CK2α. This point of view was further supported by kinetic experiments run with CK2 holoenzyme by increasing NBD1 concentration either in the absence or in the presence of the stimulatory peptides CFTR 500–518 wild type and ΔF508. As shown in Figure 6A, at all concentrations tested, holoenzyme-induced phosphorylation of NBD1 alone is negligible as compared to phosphorylation evoked by the addition of CFTR peptides, being even less pronounced than autophosphorylation of CK2 at its β-subunit. This latter is reduced or even suppressed by the addition of peptides (Figure 6B) with a concomitant striking increase in catalytic activity that highlights a positive cooperative effect of NBD1 (Figure 6B). Such behavior, revealed by the sigmoidicity of the curve, is especially pronounced if the stimulatory peptide bears the F508 deletion. In this case, by raising the NBD1
FIGURE 5: Variable modulation of CK2 holoenzyme (A) and CK2α (B) by wild-type and ΔF508 mutated NBD1 500–518 peptides. Evidence for an allosteric mechanism (C). Phosphorylation conditions by CK2α catalytic subunit and by the in vitro reconstituted holoenzyme are described in the Experimental Procedures. The substrate concentrations were 3.5, 5, 2.5, 0.5, and 100 µM for NBD1, calmodulin (CaM), inhibitor-2 of protein phosphatase 1 (I-2), HSP90, and peptide RRRADDSDDDDD, respectively. The data represent the means of at least three independent experiments with SD never exceeding 15%. (A) Effect of NBD1 peptides (80 µM) on the CK2 holoenzyme-mediated phosphorylation of the indicated substrates. (B) Effect of increasing concentrations of NBD1-derived peptides (as indicated) on CK2α catalytic subunit activity toward the indicated substrates. (C) Kinetic analysis of the mechanism of inhibition of CK2α by CFTR 500–518 ΔF508. Kinetics of the specific CK2 peptide substrate (RRRADSSDDDDD) phosphorylation by CK2α were either in the absence (■) or in the presence of CFTR 500–518 ΔF508 peptide at either 10 µM (▲) or 30 µM (▼).
concentration up to 7.5 µM, the phosphorylation rate reaches a value about 5-fold higher than in the presence of the wild-type F508-bearing peptide. This rate (about 6 nmol of P·min⁻¹·mg⁻¹ of CK2 holoenzyme) is comparable to those of typical CK2 substrates, notably calmodulin tested under identical conditions (about 8 nmol of P·min⁻¹·mg⁻¹ of CK2; see Figure 6D). In contrast, at low NBD1 concentration (2 µM or less) the phosphorylation rate in the presence of the wild-type peptide is actually higher than that observed with the ΔF508 peptide. Similar results were obtained if human instead of mouse NBD1 was used as phosphorylatable substrate (Figure 6C) although in this case the phosphorylation rate was about 3-fold lower and in the absence of peptides it was not detectable at all (not shown). Note that cooperativity was absent or modest when kinetics were run with the isolated catalytic subunit or with the holoenzyme activated by polylysine rather than by the CFTR peptides (see Figure 1). No cooperativity at all was observed if calmodulin was the substrate of CK2 holoenzyme activated by either polylysine (not shown) or the CFTR 500–518 ΔF508 peptide (Figure 6D). Collectively taken, these results provide the evidence that cooperative phosphorylation by CK2 is a unique property of CFTR NBD1, requiring the heterotetrameric structure of CK2 holoenzyme and promoted by fragments encompassing the CFTR 500–518 segment, with special reference to those bearing the F508 deletion.

To provide an independent line of evidence that NBD1, and disease relevantly, its ΔF508 mutant, may exert an allosteric regulation of CK2, their ability to physically interact with CK2 subunits was assessed by the SPR technique using surface plasmon resonance. In Figure 7 the sensograms reflecting the interactions of NBD1 with the α and β CK2 subunits, respectively, are presented. They demonstrate that although both subunits interact with NBD1, either wild type or ΔF508, in both cases the mutant binds better than wild type, the tightest interaction being the one between NBD1 ΔF508 and CK2 α-subunit. As reported in Table 1 this interaction is characterized by a quite low $K_D$.
Table 1: Binding Constants of Wild-Type and Mutated NBD1 for CK2α Catalytic Subunit

| analyte    | $k_a$ (M$^{-1}$ s$^{-1}$) $\times 10^3$ | $k_d$ (s$^{-1}$) $\times 10^{-3}$ | $K_D$ (nM) |
|------------|----------------------------------------|---------------------------------|------------|
| NBD1 wt    | 0.135                                  | 0.335                           | 2470.0     |
| NBD1 ΔF508 | 0.961                                  | 0.0877                          | 91.2       |
| Δ wt       | 22.7                                   | 0.93                            | 40.8       |

$^a$ BIAcore analysis of NBD1 wt and NBD1 ΔF508 to CK2α coupled to the biosensor surface was performed as described in the Experimental Procedures. The Langmuir 1:1 model was used to fit kinetic data. Association rates ($k_a$), dissociation rates ($k_d$), and dissociation constants ($K_D = k_d/k_a$) are reported. β interaction values were from ref 29.

![Figure 8: Effect of NBD1-derived synthetic peptides on the interaction between CK2α and CK2β subunits. 22 nM CK2α subunit was injected at a flow rate of 10 µL/min over a sensor surface containing 1660 RU of immobilized CK2β. The same amount of CK2α was injected after an incubation of 10 min with a fixed amount (5 mM) of CFTR peptide 506–518 ΔF (A) and its shortened derivative 509–518 (B). Similar results were obtained with different concentrations of CK2α.](image)

**DISCUSSION**

A priori the main if not the only biochemical argument suggesting a link between CF and protein kinase CK2 was the presence in the NBD1 domain of CFTR of a phospho-

![Table 2: Potential Sites for CK2 Phosphorylation within the Human CFTR Sequence](image)

An acidic residue at position n + 3 was chosen as a minimum requirement to identify bold-type CK2 potential sites (19). Acidic determinants are underlined.

![Figure 8](image)
phorylation of S511 despite its both conforming to the consensus sequence of CK2 and occupying an exposed position in NBD1 (11).

The mode of NBD1 phosphorylation by CK2 is also noteworthy: instead of being stimulated by the β-subunit as in the case of the vast majority of CK2 substrates, phosphorylation of NBD1 is actually fully prevented by the β-subunits, so that CK2 holoenzyme composed of two catalytic and two β-subunits (which is the predominant form of CK2 within the cell) is unable to phosphorylate NBD1 unless its activity is artificially triggered by the addition of polylysine. This behavior is not unique to NBD1, being also shared by a small subset of substrates, sometimes referred to as “class II”, typically represented by calmodulin (20). It is generally accepted that these substrates are particularly susceptible to downregulation by CK2β, which in the case of other substrates is instead overcome by concomitant upregulation by the β-subunit, whose dual function was recognized early (35, 36). Interestingly, the very same peptides encompassing the F508 region of CFTR, which proved unable to undergo phosphorylation, are nevertheless able to overcome the inhibition by β-subunit, thereby evoking NBD1 phosphorylation by CK2 holoenzyme. Our crucial result is that such a stimulatory efficacy is increased by the deletion of F508 in a fashion which is consistent with an allosteric cooperative effect on CK2 holoenzyme activity. Higher efficacy of ΔF508 peptides as compared to wild-type ones as CK2 holoenzyme activators is also consistent with the higher affinity binding to CK2 subunits of NBD1 ΔF508 as compared to wild type, as revealed by SPR experiments (see Figure 7 and Table 1). The capability of these surface-located peptides derived from NBD1 of CFTR to finely tune CK2 targeting is highlighted by the divergent effects they exert on the isolated catalytic subunit of CK2 as compared to CK2 holoenzyme and by their dependence on the nature of the phosphorylatable substrate (see Figure 5). It should be mentioned in this respect that, although the in vivo occurrence of reversible CK2 holoenzyme dissociation is still a matter of conjecture, the presence of CK2 catalytic subunits not combined with the β-subunits in different kinds of cells has been unambiguously proven (e.g., ref 37).

While the mechanism(s) by which NBD1 interacts with CK2 is (are) still a matter of conjecture, we have to assume on the one hand that the NBD1 region implicated is the one encompassing F508 since the deletion of this residue dramatically enhances the binding efficiency and on the other that such a binding perturbs the interactions between CK2α and CK2β in such a way that only downregulation by the β-subunits is abrogated, while the stimulatory efficacy of this subunit is unaffected. This bipartite conclusion is supported by the experiments of Figure 5A showing that the NBD1 peptides remove inhibition but not stimulation exerted by the β-subunit on the phosphorylation of different substrates which in general is actually enhanced by the ΔF508 peptide.

While our mechanistic study reveals a scenario where the exposed loop between NBD1 helices 3 and 4 represents a CK2 docking site whose capability to recruit CK2α is enhanced by the ΔF508 mutation and correlates with increased activity of CK2 holoenzyme toward a number of its protein targets, the identity of these substrates and the physiological significance of their phosphorylation remain a matter of conjecture.

The first candidate of course would be CFTR itself which bears around 20 residues fulfilling the consensus sequence of CK2 which are spread out in nearly all its functional domains, with special reference to NBD1, the regulatory domain (R), and NBD2 (see Table 2). As mentioned above, we have found that S422 in NBD1 is readily phosphorylated by CK2 in vitro: this residue is also phosphorylated by PKA and its phosphorylation has been shown to have an effect on CFTR activity (38) and to confer order on residues 420–428 (11) collectively referred to as the “regulatory insertion”. Curiously, there are intriguing residues in CFTR which share the features for being targets of both CK2 and PKA besides S670 (see Figure 2) whose phosphorylation by PKA affects CFTR activity (39). Of special interest in this respect may be residue S813 in the R domain, one of the four major in vivo phosphosites thought to be responsible for CFTR channel activation (40). Phosphorylation of S813 by CK2 can be primed by previous phosphorylation of T816, which in turn displays an outstanding CK2 consensus (S187E189E186S185E181E180).

The F508 deletion in CFTR is highly prevalent, being carried by 1 in 25 humans of European descent. It has long been speculated that some biological advantage must have accrued in the past for carriers of this mutation. The paradox is that such an advantage cannot be easily reconciled with the apparently normal health and CFTR ion channel function observed in carriers. However, should the F508 deleted surface of the mutant CFTR have a positive role, as our data predict, then a new scenario arises. Current dogma holds that loss of F508 creates a CFTR that does not traffic normally and degrades rapidly. Thus in this view the sole impact of ΔF508 is disrupted ion transport. Our data suggest that a ΔF508 CFTR protein could regulate CK2 targeting in multiple systems. An appealing speculation would be that in CF cells, either homozygous or heterozygous for the CFTR ΔF508 gene, activation of CK2 results in the phosphorylation and upregulation of chaperone proteins committed to the folding and processing of CFTR (or other proteins). Indeed, several chaperones are among CK2 targets (19), and a number of proteins committed to CFTR processing have been recently found in a CK2β subunit interactome isolated from rat brain (41). Interestingly, the phosphorylation of HSP90 which is unaffected by the wt peptide is stimulated by the ΔF508 peptide (see Figure 5). It will be interesting to extend this analysis to other proteins involved in the processing and trafficking of CFTR, notably HSP70, calnexin, and histone deacetylase, to see if their phosphorylation by CK2 is stimulated by the NBD1 peptides described here. Also, the identification of CK2 substrates in the macromolecular complex recruited by CFTR at the cell membrane may help to understand the functional consequences of the biochemical interactions disclosed by our work. This notion has received support from recent experiments showing that an epithelial sodium channel controlled by CFTR is CK2 regulated (42).

It should be finally remembered that most of the experiments described here were performed with mouse NBD1, because its ΔF508 mutant was available to us while the human ΔF508 mutant was not. All of the critical points,
however, notably the mode of phosphorylation by CK2α and holoenzyme, identification of the phosphorylated residues, SPR monitored interaction with CK2 subunits, and susceptibility to modulation by CFTR derived peptides, were also assessed with wild-type human NBD1, obtaining substantially identical results. This outcome and the observation that the sequence of the stimulatory peptides encompassing residues 500–518 is identical in human and in mouse NBD1 corroborate the concept that the information provided with mouse CFTR also applies to human CFTR.

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