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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 ΔF508 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 ΔF508, 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 II507 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 ΔF508 CFTR peptide. SPR analysis shows that NBD1 ΔF508 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 (ΔF508), in the nucleotide binding domain-1 (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR).[1] 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 domain 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 ΔF508 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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[1] Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator protein; NBD, nucleotide binding domain; CK2, casein kinase-2.

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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 could be the observation by Treharne et al. (*18*) that F508 is in the close proximity to a candidate phosphoacceptor residue, S511, located within a consensus sequence for the protein kinase CK2. CK2 is a highly pleiotropic protein kinase which recognizes seryl and threonyl residues specified by an acidic side chain at position $n + 3$, whose seemingly endless repertoire of substrates includes many molecules implicated in protein synthesis, folding, and degradation (*19*). CK2 has a complex structure. In general, CK2 catalytic subunits (α and/or α′) are active either alone or when combined with a dimer of two regulatory β-subunits that give rise to its hetrotetrameric holoenzyme which is the most common form of CK2 found in the cell. In a few cases however, as exemplified by calmodulin, substrate phosphorylation is actually prevented by association with the β-subunits ("class II" substrates (*20*)). CK2 is invariably elevated in tumors, and it appears to play a global antiapoptotic role, suggesting that it might represent a valuable target for anticancer agents (*21, 22*). There is an unexplained excess of cancer in young CF patients (*23*).

The proximity of the putative CK2 site S511 to the crucial F508 in CFTR prompted Treharne et al. to investigate links between CK2 and CFTR functionality (*24*). Using immunohistochemistry and electrophysiology, they proposed that CK2 colocalizes with wild-type CFTR but not with its ΔF508 mutant at the apical membranes of epithelial cells. Using the single channel configuration of the patch-clamp technique, CK2 inhibitors induced prompt (within 80 s) closure of CFTR chloride channel function, but crucially, closure was only observed when CFTR was in its native "cell attached" environment (and not when excised from the cell, presumably disrupted from its normal protein associates). They also provided in vitro preliminary data showing that CK2 is able to phosphorylate NBD1 better than NBD1 ΔF508 and much less a NBD1 mutant in which S511 was mutated to alanine. All these experiments were run with CK2 holoenzyme and were not corroborated by kinetic data nor by an analysis of physical interactions between individual CK2 subunits and NBD1, either wild type or ΔF508. This prompted us to undertake an analysis of mechanist features underlying NBD1 phosphorylation by CK2. The work presented here led to the unanticipated finding that, when NBD1 is the substrate, its phosphorylation is inhibited by the β-subunits of CK2 and CK2 phosphorylates serines 422, 423, and 670, but not to any appreciable extent Ser-511. Surprisingly, synthetic peptides encompassing the 500−518 CFTR sequence are able to trigger the activity of CK2 holoenzyme toward NBD1 in an F508-dependent manner. These data disclose new and unexpected perspectives about the implication of CK2 in CF pathogenesis by invoking the notion that F508 in CFTR controls CK2 activity in a complex manner.

EXPERIMENTAL PROCEDURES

Materials. Polylysine (M_r 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*).

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 Nα-Fmoc amino acids carrying standard side chain protective groups were converted to benzotriazolyl esters with 1-hydroxybenzotriazole (HOBt) and 2-(1*H*-benzotriazol-1-yl)-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/H_2O/thioanisole/ethanedithiol/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 $MgCl_2$, 100 mM NaCl, and 100 μM [γ-^{33}P]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 DLUs and ^{33}P cpm was deduced by spotting known amounts of γ-[^{33}P]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

cpm spotted on the membrane, the amount of ^{33}P cpm per DLU was calculated and the radioactivity at selected fields assessed. Alternatively, the bands corresponding to the protein of interest on nitrocellulose membranes were excised and subjected to liquid scintillation counting.

Phosphopeptide Enrichment and MS Analysis. After in vitro phosphorylation of NBD1, the protein (8 μg) was run on a SDS−PAGE, and the gel bands were cut and subjected to reduction/alkylation and in-gel digestion using sequencing grade modified trypsin (Promega, Madison, WI). Briefly, gel bands were crushed, washed with acetonitrile, dried under vacuum, and treated with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 56 °C. The DTT solution was then removed and the gel pieces were further treated with 55 mM iodoacetamide (in 100 mM NH_4HCO_3) for 45 min in the dark. After extensive washing with 100 mM NH_4HCO_3 and acetonitrile, the gels pieces were dried and finally treated with 12.5 ng/μL trypsin at 37 °C overnight. After digestion, the peptides were extracted by three changes of 50% acetonitrile/0.1% formic acid (20 min between changes), dried under vacuum, and resuspended with 10 μL of 0.1% formic acid.

Phosphopeptides were enriched on TiO_2 microcolumns as previously described (*28*).

The phosphopeptide enriched samples were analyzed using a LCQ XP (Thermo Electron, San José, CA) interfaced with a nano-LC system 1100 series (Agilent Technologies, Santa Clara, CA) and a capillary column Zorbax 300SB C18, 3.5 μm, 150 mm × 75 μm (Agilent), using a linear gradient of acetonitrile/0.1% formic acid from 5% to 40% in 20 min. Data were acquired in a data-dependent mode: a full MS scan was followed by a Zoom scan and a MS/MS scan on the three most intense peaks. MS/MS data were searched using Mascot (Matrix Science, London, UK) against the mouse section of the IPI database (version 3.31, 56555 entries). Enzyme specificity was set to trypsin with one missed cleavage using carbamidomethylcysteine as fixed modification and phosphorylation of S/T/Y as variable modification. The tolerance of the precursor ion was set to 1 mass unit for both peptide and fragment ion matches. All of the MS/MS spectra were then carefully inspected to confirm the identifications, and many of them had to be manually interpreted to correctly assign the position of the phospho residue(s).

SPR Analysis. BIAcoreX system was used to detect and determine the kinetic constants of the interactions. CK2α and CK2β subunits were covalently linked to sensor chips CM5 (BIAcore) using amine coupling chemistry (*29*). Solutions of the interacting proteins, NBD1 wt and NBD1 ΔF508 ("analyte"), were injected with a flow rate of 10 μL/min at 25 °C in HBS running buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20). The response obtained with a control surface (without immobilized protein) was subtracted to each sensorgram. The kinetic data were interpreted according a simple 1:1 binding model, and the rate constants of the interactions were calculated using the SPR kinetic evaluation software BIAevaluation 3.0 (BIAcore).

RESULTS

The phosphorylation of mouse wild-type, mouse ΔF508, and human wild-type CFTR nucleotide binding domain 1 (NBD1) by protein kinase CK2, either catalytic subunit or reconstituted holoenzyme, is shown in Figure 1A: mouse NBD1 ΔF is phosphorylated by CK2α slightly better than its wild-type counterpart which in turn is phosphorylated more readily than the wild-type human homologue. Phosphorylation is abolished in all cases if the catalytic subunits of CK2 are combined with the β-subunits to form CK2 holoenzyme. The latter is in fact inactive toward all NBD1 forms unless polylysine is present. Polylysine overcomes downregulation by the β-subunit and stimulates NBD1 (wild type and ΔF508) phosphorylation by the holoenzyme beyond the level observed with the catalytic subunit alone. Addition of polylysine to the α-subunit alone has no significant effect (not shown). Kinetic analyses of murine wild-type vs ΔF508 NBD1 phosphorylation by CK2 α-subunit show that they display a similar behavior (Figure 1B). Kinetics performed with CK2 holoenzyme plus polylysine (Figure 1C) are also

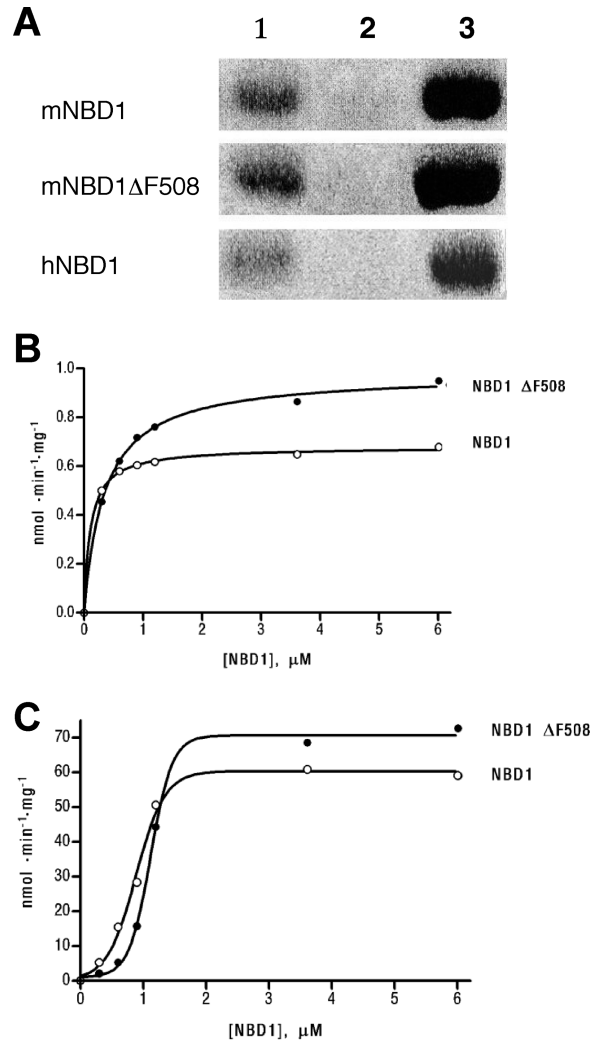

FIGURE 1: Phosphorylation of wild-type and ΔF508 mutated NBD1 by protein kinase CK2. In (A) 3 μg of murine and human NBD1 were incubated under conditions decribed in the Experimental Procedures with CK2α catalytic subunit either alone (lane 1) or previously combined with equimolar amounts of CK2β regulatory 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 (○) and ΔF508 (●) are illustrated. The data represent the means obtained from experiments run in triplicate with SD never exceeding 15%.

A

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*DENNVSF**
720.44	2	1438.51	0.35	**HS*SDENNVSFSH**
760.38	2	1518.48	0.26	**HS*S*DENNVSFSH**
776.95	2	1551.60	0.29	**HS*SDENNVSFSHL**
785.29	2	1567.59	0.97	**KHS*SDENNVSFSH**
834.46	2	1646.58	0.34	**KHS*S*DENNVSFSH**
824.45	2	1646.58	0.32	**KHS*S*DENNVSFSH**
840.65	2	1679.69	-0.41	**KHS*SDENNVSFSHL**
561.05	3	1679.69	0.42	**KHS*SDENNVSFSHL**
880.59	2	1759.72	-0.54	**KHS*S*DENNVSFSHL**
1331.61	2	2660.24	0.95	**KHS*SDENNVSFSHLCLVGNPVLK**
914.81	3	2741.19	0.21	**KHS*S*DENNVSFSHLCLVGNPVLK**

B

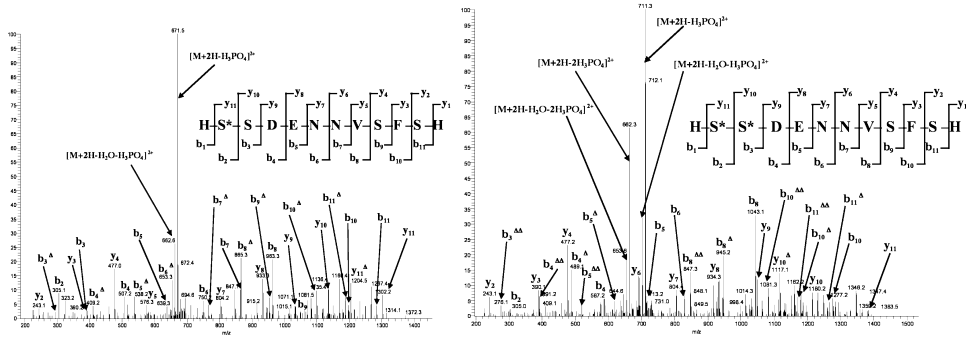

FIGURE 2: MS analysis of NBD1 phosphorylation sites by protein kinase CK2. Phosphorylation of mouse wild-type NBD1 by CK2 holoenzyme in the presence of polylysine and elution of the radiolabeled bands from the SDS−PAGE gel were performed as described in the Experimental Procedures. (A) List of identified 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 phosphoresidue 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 Δ indicates the fragments originated by the neutral loss of phosphate.

similar with either NBD1 wild type or ΔF508. Note however that the apparent K_m values in this case are higher than those calculated with CK2α, 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 ΔF508 were exaustively phosphorylated by CK2 holoenzyme in the presence of polylysine, and the phosphorylated residue(s) was (were) identified by tryptic digestion followed by MS analysis as detailed in the Experimental Procedures. The results summarized in Figure 2 led to the unambiguous identification of peptides including three phosphorylated residues: pS422, either alone or together with pS423, and pS670. Both S422 and S670 display the typical CK2 consensus, while S423 does not. Note however 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 ΔF508 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-

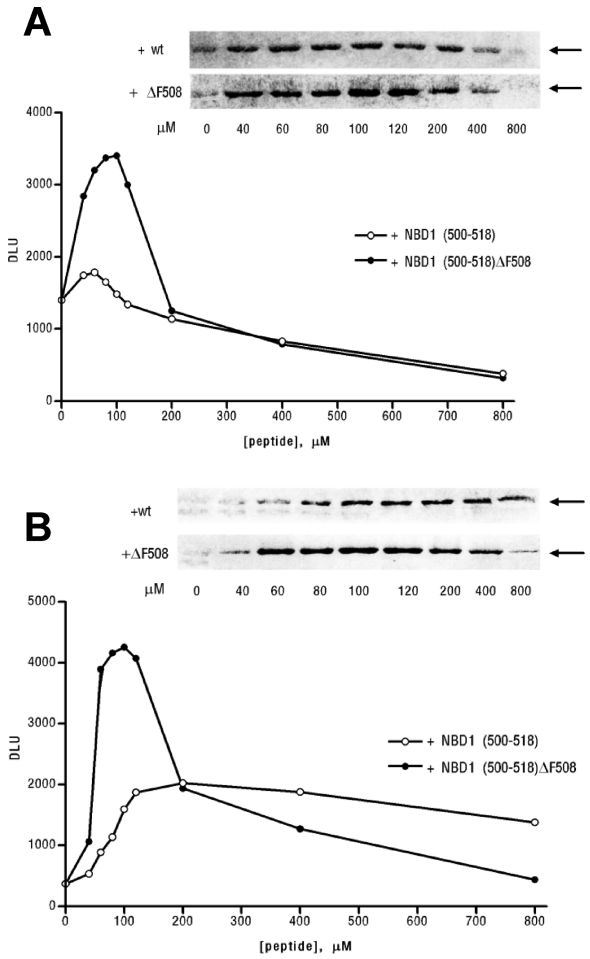

FIGURE 3: Dose-dependent effect of NBD1-derived synthetic peptides on the phosphorylation of NBD1 by protein kinase CK2. Phosphorylation of wild-type murine NBD1 (3 μg) was performed by CK2 catalytic subunit (panel A) and by CK2 holoenzyme (panel B) as described in the Experimental Procedures in the presence of increasing concentration of the NBD1-derived wild-type GTIKENIIFGVSYDEYRYR (○) and ΔF508 mutated GTIKENIIGVSYDEYRYR (●) 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%.

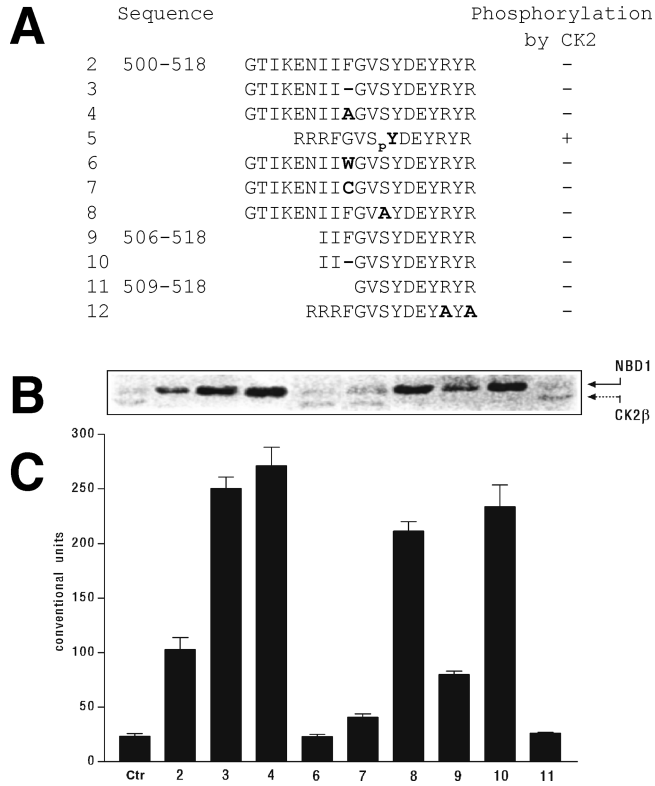

FIGURE 4: Effect of variable substitutions within the NBD1-derived peptides on their stimulatory efficacy on NBD1 phosphorylation by CK2 holoenzyme. Murine NBD1 (3 μg) was phosphorylated by CK2 holoenzyme under conditions described in the Experimental Procedures in the presence of variably substituted 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).

rylated S511, conforming to the CK2 consensus sequence (S^{511}YDE), 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 CK2α 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 CK2α 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

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 GTIKENIIFGVSYDEY-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 IIF^{508} 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 ($R_3AD_2SD_5$). 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

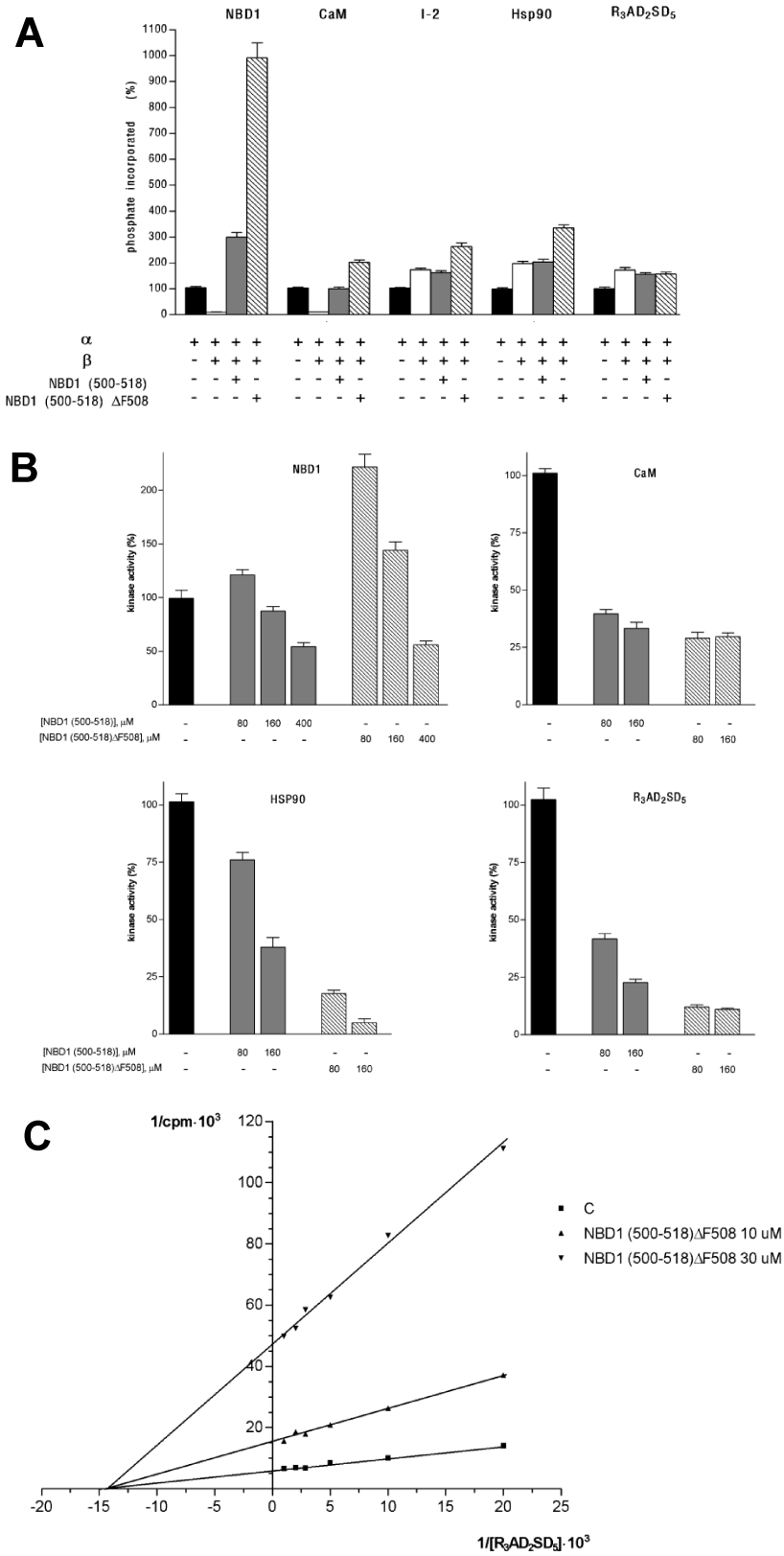

FIGURE 5: Variable modulation of CK2 holoenzyme (A) and CK2α (B) by wild-type and ΔF508 mutated NBD1 500−518 peptides. Evidence for an allosteric mechanism (C). Phosphorylation conditions by CK2α catalytic subunit and by the in vitro reconstituted holoenzyme are described in the Experimental Procedures. 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 (RRRADDSDDDDD) 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 (▼).

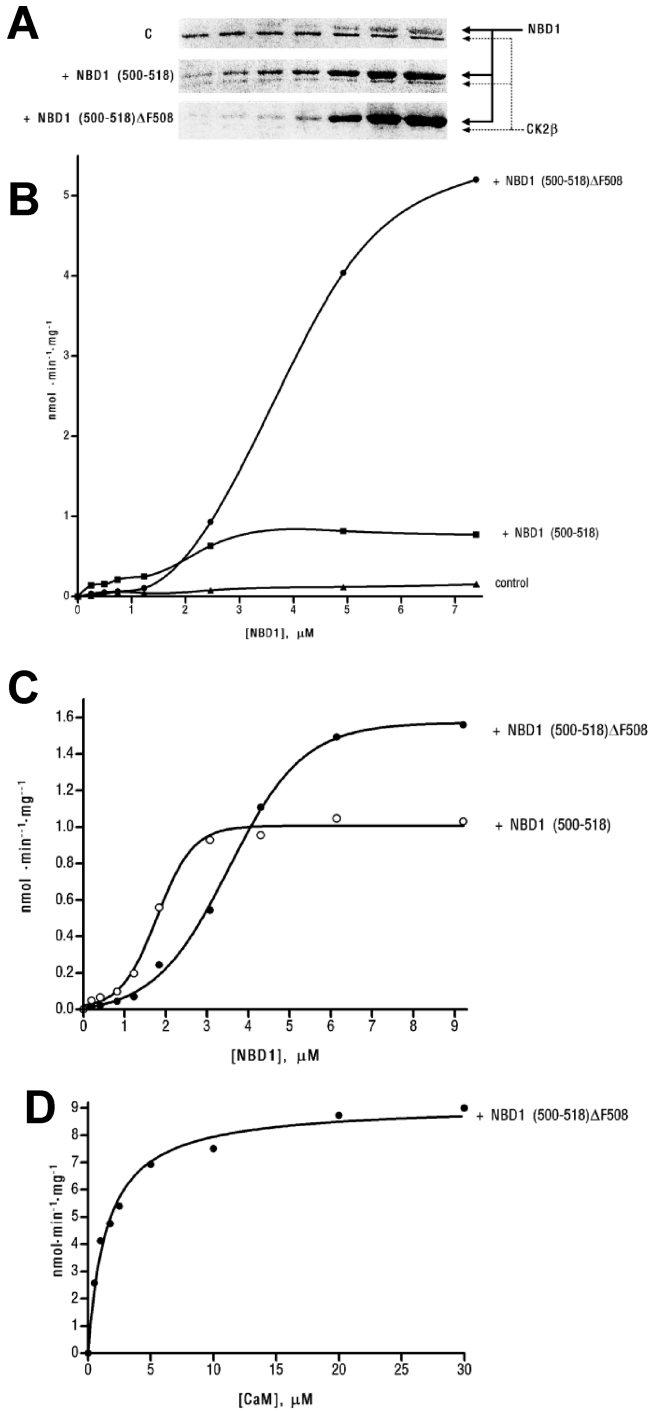

FIGURE 6: Positive cooperative effect of NBD1 phosphorylation by CK2 holoenzyme in the presence of wild-type and ΔF508 peptide. Phosphorylation condition at increasing concentration of the substrate and evaluation of the phosphate incorporated after SDS−PAGE were performed as described in the Experimental Procedures. The data represent the means obtained from experiments run in triplicate with SD never exceeding 20%. (A, B) Murine NBD1 either alone (control) or in the presence of wild-type and ΔF508 mutated peptides (80 μM). The corresponding autoradiograms are shown in (A). (C) Human NBD1 in the presence of wild-type and ΔF508 mutated synthetic peptides (80 μM). (D) Calmodulin in the presence of ΔF508 mutated peptide (80 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 $P \cdot min^{-1} \cdot mg^{-1}$ of CK2 holoenzyme) is comparable to those of typical CK2 substrates, notably calmodulin tested under

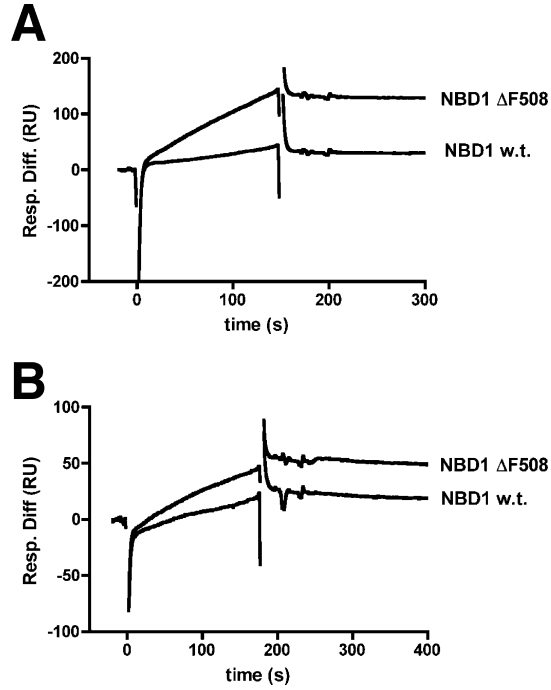

FIGURE 7: SPR analysis of the interaction of mouse NBD1 wild type and ΔF508 with human CK2 subunits. Representative sensorgrams obtained as detailed in the Experimental Procedures by injection of 15 μM NBD1 wt and NBD1 ΔF508 over a sensor surface containing 1600 RU of immobilized CK2α (A) and of 10 μM NBD1 wt and NBD1 ΔF508 over a sensor surface containing 1660 RU of immobilized CK2β (B).

identical conditions (about 8 nmol of $P \cdot min^{-1} \cdot mg^{-1}$ 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 sensorgrams 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[a]

analyte	k_a (M^{-1} s^{-1}) × 10^3	k_d (s^{-1}) × 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*.

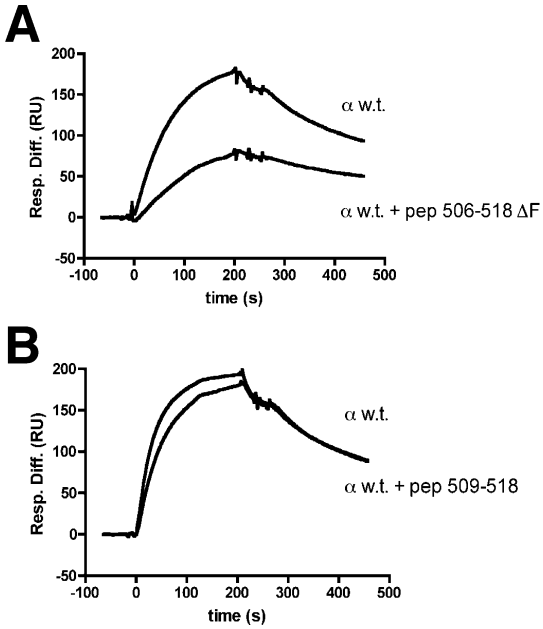

FIGURE 8: Effect of NBD1-derived synthetic peptides on the interaction between CK2α and CK2β subunits. 22 nM CK2α subunit was injected at a flow rate of 10 μL/min over a sensor surface containing 1660 RU of immobilized CK2β. The same amount of CK2α was injected after an incubation of 10 min with a fixed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

value (91.2 nM) which is comparable to the one calculated for the β-subunit itself (40.8 nM) whose complex with CK2α is extremely stable in vitro, requiring denaturing conditions for dissociation (*34*). In contrast, the interaction of NBD1 wild type with CK2α is significantly weaker (K_D = 2470 nM), the difference being mainly accounted for by an almost 1 order of magnitude lower association constant ($k_a = 0.135 \times 10^3$ vs 0.96×10^3). This suggests that association with CK2α involves the NBD1 segment affected by the ΔF508 mutation. Such a conclusion was strengthened by the experiment illustrated in Figure 8, showing that the interaction between CK2 β- and α-subunits is weakened by the CFTR peptide 506−518 ΔF, which also promotes NBD1 phosphorylation by CK2 holoenzyme but not by its shortened derivative 509−518 which has lost the ability to evoke CK2 holoenzyme activity (see Figure 4).

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 phosphoacceptor residue (S511) fulfilling the CK2 consensus (S^{511}yDEyr) in the close proximity of F508, i.e., the residue whose single deletion (ΔF508 or F508del) is the by far the most common cause of cystic fibrosis affecting around 75% of diseased chromosomes. F508 is outside the *sensu stricto* CK2 site (conventionally specified by acidic residues at positions between $n - 2$ and $n + 5$ (*19*)), and its deletion was not predicted to necessarily affect the proneness of S511 to CK2-mediated phosphorylation. Nevertheless, it is quite reasonable to speculate that the deletion of such a bulky hydrophobic side chain at position $n - 3$ with respect to the target residue might have consequences on the phosphoacceptor site conformation and therefore on its phosphorylation efficiency by CK2.

Table 2: Potential Sites for CK2 Phosphorylation within the Human CFTR Sequence[a]

sequence	CFTR domain	conserved in murine CFTR
MQR**S$_4$**PLEKASV	intracellular domain	yes
AVQ**T$_{360}$**WYDSLGA	intracellular domain	no
YNL**T$_{390}$**TTEVVME	NBD1	no
RKT**S$_{422}$**NGDDSLF	NBD1	yes
MPG**T$_{501}$**IKENIIF	NBD1	yes
FGV**S$_{511}$**YDEYRYR	NBD1	yes
DVL**T$_{582}$**EKEIFES	NBD1	no
LVT**S$_{605}$**KMEHLKK	regulatory domain	yes
FYG**T$_{629}$**FSELQNL	regulatory domain	no
ANL**T$_{803}$**ELDIYSR	regulatory domain	no
RRL**S$_{813}$**QE**T$_{816}$**GLEISEEINEED	regulatory domain	no
LPL**T$_{990}$**IFDFIQL	intracellular domain	yes
SIL**T$_{1121}$**TGEGEGR	intracellular domain	yes
AVN**S$_{1149}$**SIDVDSL	intracellular domain	yes
GQM**T$_{1211}$**VKDLTAK	intracellular domain	no
LLN**T$_{1263}$**EGEIQID	NBD2	no
GLR**S$_{1327}$**VIEQFPG	NBD2	yes
QAI**S$_{1443}$**PSDRVKL	intracellular domain	yes
KEE**T$_{1472}$**EEEVQDT	intracellular domain	yes

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

The results presented corroborate the concept introduced by Treharne and colleagues that functional links indeed exist between CK2 and CFTR which are deeply affected by the F508 deletion. Somewhat paradoxically however our findings tend to exclude any phosphorylation of S511 by CK2 while supporting the view that the sequence surrounding F508 represents, with respect to CK2, at the same time a docking site and an allosteric effector whose dual function is increased by F508 deletion.

Although in fact NBD1 is readily phosphorylated by CK2 catalytic subunit and even more by CK2 holoenzyme in the presence of polylysine, S511 could not be found among the phosphoresidues identified by MS analysis: these are S422 (either alone or together with S423) and S670, both fulfilling the consensus sequence for CK2 and conserved in human CFTR as well (see Table 2). Failure of CK2 to phosphorylate S511 was also confirmed by using a set of peptide substrates variably reproducing the sequence around this residue: the phosphorylation of all peptides either with naturally occurring sequences or with suitable modifications was null or anyway negligible as compared to that of full-length NBD1, with the only partial exception of a phosphopeptide in which Y512 was replaced by phosphotyrosine to mimic a hypothetical priming effect of Syk tyrosine kinase. Thus it is not clear which are the negative determinant(s) preventing the phos-

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 (S^{813}qET^{816}glEsEEinEED).

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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