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CFTR FRAGMENTS WITH THE F508 DELETION EXERT A DUAL ALLOSTERIC CONTROL OVER THE MASTER KINASE CK2.

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¹ Abbreviations: CFTR, cystic fibrosis trans membrane regulator; NBD1, nucleotide binding domain 1; CK2, casein kinase 2.

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SUMMARY

Cystic Fibrosis (CF) mostly follows a single F508 deletion in CFTR (Δ F508), thereby causing premature fragmentation of the nascent protein with concomitant alterations of diverse cellular functions. We show that CK2, the most pleiotropic protein kinase, undergoes allosteric control of its different cellular forms in the presence of short CFTR peptides encompassing the F508 deletion: these Δ F508 peptides drastically inhibit the isolated catalytic subunit (α) of the kinase and yet up-regulate the holoenzyme, composed of two catalytic and two non catalytic (β) subunits. Remarkable agreement between *in silico* docking and our biochemical data point to different sites for CFTR- Δ F peptide binding on isolated CK2 α and on CK2 β assembled into the holoenzyme suggesting that CK2 targeting may be perturbed in cells expressing Δ F508CFTR; this could shed light on some pleiotropic aspects of CF disease.

Keywords

Cystic fibrosis; protein kinase CK2; CK2 allosteric regulation; delta F508 CFTR fragments; CFTR peptides; molecular modelling.

INTRODUCTION

Eukaryotic life is controlled by the reversible phosphorylation of proteins such that ~50% of the human proteome (also referred to as the “phosphoproteome”) is phosphorylated at some time [1, 2]. Such phosphotransferase reactions are catalyzed by protein kinases, making up one of the largest families of enzymes, collectively referred to as the “kinome” [3]. The specific contribution of the individual members (>500 in the human kinome) is non linear such that a few are dedicated enzymes impinging only on a paucity of protein targets whereas the majority are more or less pleiotropic with many protein substrates subjected to their control. Both based on available repertoires of substrates [4] and on a global phosphoproteome analysis [5], one single protein kinase is endowed with the most striking pleiotropy, possibly accounting for the generation of 10-20% of the entire phosphoproteome. This protein kinase is CK2, an acronym derived from the misnomer “casein kinase-2” applied pragmatically merely to indicate one of the ubiquitous acidophilic Ser/Thr protein kinases displaying high *in vitro* activity toward the artificial substrate casein even though this is not among its many physiological targets [6]. CK2 appears to be endowed with an ample variety of cellular functions, with special reference to gene expression, protein synthesis and degradation and signal transduction [7] and plays a key role as a pro-survival and an anti-apoptotic agent [8]. In particular it has been suggested that the critical role of CK2 in the NF- κ B and in the Wnt pathways [9] accounts for the death at mid gestation of mice lacking the catalytic subunit α of CK2 [10]. While loss of this CK2 catalytic subunit in development is lethal, its overexpression is capable of promoting tumorigenesis [11-14] and, more in general, alterations in CK2 level have been linked to a variety of pathological situations [15].

It is commonly held that the pathogenic potential of CK2 is related to its constitutive activity, which, in turn, may reflect its terrific pleiotropy [16]. However, this notion does not sit well with the fact that CK2 is equipped with a regulatory (β) subunit whose obligate dimer status creates the backbone to form a heterotetrameric holoenzyme once combined with two catalytic subunits (α and/or α'). These latter catalytic moieties are active either alone or in combination with the β subunits, with the caveat that the regulatory subunits appear to display a variable influence on the targeting of different substrates [17] despite themselves being unable to turn catalytic activity either on or off. This creates 3 different classes of CK2 substrates depending on how β binding affects their phosphorylation by α . Consistent with the concept of CK2 being constitutively active lies the observation that in all CK2 α structures solved to date, regardless of species, crystal packing, ligands and quaternary structure, the regulatory key elements invariably adopt the conformation typical for active protein kinases [18]. Recently, a careful inspection of CK2 α disclosed the existence of some dynamic regions therein [19], one of which has the potential to overlap a proposed binding pocket for small molecules located at the interface with the β -subunit [20]. It has been speculated that this represent an allosteric site where some recently developed non competitive inhibitors of CK2 holoenzyme may bind [21]. On the other hand no physiological effector(s) capable of exploiting this putative, down-regulatory allosteric site are known. Recently, however, we reported that peptides encompassing the sequence of cystic fibrosis transmembrane regulatory protein (CFTR) surrounding F508, a residue whose deletion is the commonest cause of cystic fibrosis, are powerful effectors of CK2 activity displaying opposite effects on the holoenzyme as compared to the isolated catalytic subunits [22]. In particular peptides bearing the F508 deletion (Δ F peptides) were able to specifically inhibit CK2 α in a non-competitive manner with respect to a specific phosphoacceptor substrate. Recently, Cohen et al. [23] described *in vivo* effects of these very same peptides in a developing lung model such that F508-dependent changes in protein-protein interactions occur when such peptides are expressed using a peptide-encoding virus to infect the developing embryo. Importantly, in cystic fibrosis (CF), the mechanism by which loss of F508 induces a multi-system disease that destroys many organs remains unknown. Here, we investigate the optimal features

conferring inhibitory properties to these peptides. We show that they behave as allosteric effectors of CK2 by binding to two distinct sites, one of which may be located in a region of the upper lobe of the α subunit where it can both interfere with α/β inter-subunit interaction altering the proper conformation of the ATP binding domain. Our work may have immediate relevance to the amplification mechanism by which a single deleted amino acid in CFTR could cause so dramatic a change in the phenotype in ~ 1 in 3000 human infants creating a multi-organ disease affecting networks of many hundreds of proteins and genes.

EXPERIMENTAL

Materials

Most of the reagents were purchased from Sigma. Purified wild type and $\Delta F508$ mutated recombinant murine NBD1 (spanning sequence 389-673) were generously provided by the Philip J. Thomas laboratory (Southwestern Medical Center, University of Texas, Dallas TX, USA; <http://www4.utsouthwestern.edu/thomaslab/>). Recombinant α and β subunits of human protein kinase CK2 were expressed in *E. coli* and purified as previously described [24].

Peptide synthesis

The CFTR-derived synthetic peptides were prepared by solid phase peptide synthesis method using an automatized peptide synthesizer (model 431-A, Applied Biosystems, Foster City, CA) or/and a multiple peptide robotic synthesizer (model Syro II, MultisynTech, Witten, Germany) as C-terminal acids on HMP resin (Applied Biosystems) or as C-terminal amides on Rink Amide PEGA resin (Novabiochem, Bad Soden, Germany) as previously described [22].

Phosphorylation assays

In vitro phosphorylation experiments were performed by incubating CK2 α catalytic subunit (8-28 nM) with peptide or protein substrates (final volume 25 μ l) in a medium containing 50 mM Tris-HCl pH 7.5, 12 mM MgCl₂, 100 mM NaCl, 100 μ M [γ -³³P]ATP (specific radioactivity 2000-4000 cpm/pmol). The synthetic peptide RRRADDSDDDDD (100 μ M) was used as a typical CK2 α phosphoacceptor substrate while a peptide derived from eIF2 β [25] and calmodulin were also used in some experiments for other class effects. The autophosphorylation of CK2 β subunit was evaluated by incubating the CK2 holoenzyme in the presence of the phosphodonor nucleotide as described above and by subjecting samples to SDS-PAGE. The phosphate incorporated into peptide and protein substrates was determined as previously described [25, 22]. Kinetic analysis and determination of inhibition constants were performed by testing the kinase in the absence or in the presence of increasing concentrations of inhibitor and by constructing the Lineweaver-Burk double-reciprocal plots of the data. Final data reported in Figures and Tables are calculated from the mean of at least three independent experiments with SEM never exceeding 15%.

Molecular modelling

For the computer aided protein-protein docking stages human CK2 α subunit, the human CK2 $\alpha_2\beta_2$ tetramer and CFTR ΔF peptide (AA500-518) were retrieved from the PDB (PDB code: 1JWH and 1XMJ, respectively). Hydrogen atoms were added to the protein structure using standard geometries with the MOE program [26]. To minimize contacts between hydrogens, the structures were subjected to Amber99 force field minimization until the rms of conjugate gradient was $<0.1 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ keeping the heavy atoms fixed at their crystallographic positions. The wt

CFTR, CFTR Δ F and CFTR Δ I peptides were built starting from the crystallographic structure 1XMJ and submitted to MOE molecular dynamic approach for 1ns at a constant temperature of 300K within a previously minimized water system [26]. After this initial in silico approach a set of protein protein docking experiments was performed. Protein–protein docking is a computational tool useful to predict the 3D structure of protein complexes from the coordinates of its subunits. We have adopted a two-stage approach, which recently demonstrated good efficacy; in the first stage, the two monomers was treated as rigid bodies and all the rotational and translational degrees of freedom was fully explored, using a cluster of protein protein docking algorithms (Zdock, Gramm and Echer); in the second stage, a small number of structures deriving from the initial stage was refined in the docking zone using side-chain rotamers and an energy-minimization strategy [27, 25]. The final complexes was submitted to a new molecular dynamic step at the same condition described before to validate their stability over time.

RESULTS

Structural features of CK2 α inhibitory peptides derived from CFTR.

Fig. 1 illustrates the dose-dependent effect of wild-type or various mutated peptides encompassing the CFTR 500-518 sequence (see Table 1, peptide 1), on the activity of CK2 catalytic subunit (α) assayed with the alpha specific peptide substrate R₃AD₂SD₅. At low CFTR-peptide concentrations, inhibition by the wild type peptide (open circles) is not apparent (being rather replaced by slight stimulation) but nonetheless, inhibition initiates at concentrations beyond 50 μ M. Deletion of F508 is itself sufficient to convert the peptide into a powerful inhibitor with IC₅₀ around 15 μ M (compare open circles with filled squares). By contrast the deletion of I507 (open squares), also responsible for CF on one allele by promoting unfolding and premature degradation [28], but nonetheless extremely rare (<0.1%) as compared to the F508 (70-90%) [29] rather causes a loss in inhibitory efficacy. Also of note in Fig. 1 is the effect of mutating V510 to D either in the context of a wild type peptide (diamonds) or its Δ F508 equivalent (filled circles), the latter combination having been shown to partially restore functionality of CFTR Δ F508 [30]: in our hands this V510D mutation significantly abrogates the inhibitory efficacy of the Δ F508 peptide (compare filled squares with filled circles), yet has a diametrically opposed effect when present in the wild type peptide whose moderate inhibitory efficiency is increased almost equalling that of the Δ F508 peptide (compare open circles with diamonds). These data demonstrate that the sequence around the common mutation in CFTR has residue-dependent regulatory relevance to CK2 α function. Recently, Treharne et al. have demonstrated that CK2 can control CFTR itself in an F508-related manner, thus adding further evidence to our proposed link between CFTR and CK2 [31] through this F508 region.

The peptides used for the experiment in Fig. 1 encompass a region of CFTR NBD1 centred on an exposed loop bridging two helices [32], also including two short “wings” belonging to these helices (see scheme above Table 1). To gain information about which elements within the peptides might be important for their efficacy towards CK2 α activity, shortened derivatives were synthesized bearing deletions on their N and/or C-termini. These are listed together with the “parent” peptides in Table 1 where their IC₅₀ values, reflecting the ability of each to inhibit CK2 α are also reported. These data lead us to conclude that while the first 6 N-terminal residues (GTIKEN) are dispensable, as their removal does not compromise inhibition by either the wild type or the Δ F peptides (compare peptides 1 and 2 with peptides 7 and 11, respectively) any further removal of the II doublet is dramatically detrimental as it fully abrogates their inhibitory potential (compare peptide 2 with peptide 3). This is consistent with the crucial role of I507 whose deletion is sufficient to

suppress inhibition (see Fig. 1). By sharp contrast the whole C terminal part of the peptides (generating in full size NBD1 the initiation point of a new helix) appears to be strictly required for efficient inhibition, since the removal of the C terminal doublet YR from a ΔF N-terminally shortened peptide abrogates inhibition, at least up to 250 μM (compare peptide 12 with peptide 11 in Table 1).

Kinetics are consistent with an allosteric mechanism of inhibition of CK2 α by CFTR ΔF peptides.

The CFTR peptides used in this study include a potential CK2 phosphoacceptor site (S511) which is not phosphorylated by CK2 to any appreciable extent [22]. It also includes 3 tyrosyl residues one of which (Y512) could represent a target for the tyrosine kinase Syk, owing to nearby acidic determinants [33]. We have found that the CFTR(500-518) peptide is unaffected by the Src family protein kinases Lyn and c-Fgr, while being slowly phosphorylated by Syk ([34] and unpublished data) and that a derivative of this peptide in which Y512 was replaced by phosphotyrosine underwent a weak phosphorylation by CK2 at S511 [22]. We wondered whether the inhibition of CK2 α by these peptides was due to a pseudosubstrate effect, resulting in competition with respect to the phosphoacceptor substrate. To check this point and, more in general, to gain information about the mechanism underlying inhibition of CK2 α by CFTR peptides, competition kinetics were performed with respect to either the phosphoacceptor substrate or the co-substrate ATP. It was previously found that inhibition of phosphorylation by the ΔF peptide was not competitive with respect to the specific CK2 peptide substrate R₃AD₂SD₅ [22]. Similar results have been now obtained using the more physiological target calmodulin as phosphoacceptor substrate (Fig. 2A): again inhibition by ΔF CFTR peptide is not competitive with respect to this phosphoacceptor substrate. On the other hand, competition experiments with respect to the co-substrate ATP (Fig. 2B) gave rise to bi-phasic kinetics: at concentrations around its K_m value (15 μM), which are those generally adopted for this kind of kinetics, ATP is typically non competitive against inhibition by the CFTR peptide, with V_{max} values drastically diminished by the CFTR peptide, while K_m values are similar; if however ATP concentration is increased above 100 μM , this tends to attenuate inhibition, disclosing a mixed mechanism of inhibition: the double reciprocal plot of this part of the curve reveals both increased K_m and decreased V_{max} values, suggesting that the binding sites for the peptide and for ATP, albeit physically separated, are functionally linked. These data are consistent with an allosteric mechanism of inhibition whereby upon binding of the CFTR peptide the affinity for ATP is drastically decreased. We also observe that the inhibitory efficacy of the peptide can be overcome by ATP only if the peptide concentration is much lower than its IC_{50} value (2.5 μM versus 15 μM): with 10 μM peptide in fact the V_{max} remains 2.5-fold lower than in its absence, showing that under these conditions the catalytic mechanism is impaired even if the ATP concentration is raised to saturating level.

Inhibition of CK2 α by CFTR ΔF peptide is abrogated by CK2 β -subunit and by full length NBD1.

Since the inhibitory effect of the CFTR peptides was not observed when the specific peptide substrate was phosphorylated by CK2 holoenzyme [22] we reasoned that the peptide binding site could be located on the surface of CK2 α facing the β -subunit where it may no longer have been accessible once the heterotetrameric holoenzyme had formed.

This point was further investigated by evaluating the inhibitory efficacy of increasing amounts of the ΔF peptide either directly on CK2 α or on CK2 α previously preincubated with 10-fold molar excess β -subunit: as shown in Fig. 3A pre-treatment with β fully protects α from the inhibitory effect of the ΔF 508-CFTR peptide. This is consistent with the idea that the CK2 α site, where the ΔF 508-CFTR peptide exerts its down-regulation on the catalytic subunit in isolation, is now either occupied by β or is, in some unknown manner, no longer accessible once β is bound to α (note that

the peptide also binds the β subunit, see below). The interactions occurring between CK2 subunits are known from the crystal structure of CK2 holoenzyme [35]: in particular an exposed region of the upper lobe of the α subunit is implicated which makes multiple contacts with diverse elements within the β subunits. Especially important in this respect appears to be the C-terminal segment of the β -subunit encompassing residues 182 to 203: a peptide reproducing this region was previously shown to interact with the N-terminal region of the α subunit [36] and to partially mimic some of the effects of the full size β subunit [37]. We therefore took advantage of the same peptide to check if it might compete against inhibition by the ΔF peptide. As shown in Fig. 3B the β peptide (onset at 50 μM) by itself exerts a modest stimulation of CK2 α catalytic activity, as previously observed [37]. Albeit starting from a lower baseline, such a stimulatory effect is much more pronounced if the experiment is performed with CK2 α pre-inhibited by the concomitant presence of the ΔF peptide. As a result (Fig. 3C) inhibition by the ΔF peptide is significantly reduced by increasing concentrations of the β -peptide, consistent with competition between the two peptides for the same or overlapping site(s). This permitted us to map the allosteric site to the upper lobe of CK2 α , precisely to the β strands 1 and 2. Note that the relatively modest effect of the β peptide as compared to full length β closely reflects the different affinity for α showing a K_D value of 8.45 μM and 40 nM, respectively ([22] and Table S1 of Supplementary material). Very high affinity of β for α may also account for the fact that the ΔF CFTR peptide fails to dissociate the holoenzyme, as revealed by size exclusion chromatography experiments revealing no detectable generation of isolated CK2 subunits from the holoenzyme treated with 100-fold molar excess of either the usual CFTR ΔF (500-518) peptide (Supplementary Material Fig. S1 B) or a longer version of it (500-523) characterized by higher water solubility (not shown). The observation however that treatment with either peptides results in broadening of the peak corresponding to the holoenzyme (compare panels A and B in Fig. S1) suggests that the peptide affects the overall conformation of the tetramer, possibly weakening the inter-subunit interactions (as previously observed by SPR experiments using a similar ΔF peptide [22]).

Next, we determined whether full length NBD1 competes for a peptide derived from itself. Since we have previously reported that recombinant purified full length NBD1 ΔF (gift of P. Thomas, USA) strongly interacts with CK2 α [22], a pertinent question is whether binding occurs through the structural elements reproduced by the ΔF 508 peptide (residues 500-518, Table 1). To answer this question competition experiments were designed aimed at assessing whether recombinant purified ΔF -NBD1 can overcome the inhibitory effect of a peptide reproducing its own F508-deleted 500-518 stretch. A technical problem in running this experiment arises from the fact that the concentrated stock of NBD1 is dissolved in high ATP concentration (2 mM) in order to maintain its proper conformation for folding studies, a circumstance which could lead to isotopic dilution of radiolabeled ATP in the kinase assay. To circumvent this problem the whole experiment was performed at 350 μM ATP either in the absence or presence of NBD1 ΔF . Under these conditions, phosphorylation of NBD1 ΔF was negligible as compared to that of the peptide substrate (see legend of Fig. 3); therefore substrate radiolabeling is fully accounted for by the peptide.

As shown in Fig. 3D the addition of 50 μM NBD1 ΔF abrogates the inhibitory efficacy of the ΔF peptide, consistent with the view that the protein and the peptide bind to the same CK2 site, most likely through the same structural elements considering that the F508 deletion is essential for both high affinity binding of NBD1 [22] and efficient inhibition by the peptide (see Fig. 1 and table 1). Note that the outcome of this experiment implies that the binding of full length ΔF -NBD1 is not promoting by itself any appreciable inhibition of CK2 α . This point was also independently assessed by control experiments where no significant inhibition of CK2 α could be observed by raising ΔF -NBD1 concentration up to 20 μM (not shown) a concentration sufficient to display a protective effect against inhibition by the ΔF peptide. A possible explanation for the differential efficacy of the full size protein versus the peptide derived from it could reside in the greater rigidity of the

folded NBD1 Δ F structure [32] as opposed to the Δ F peptide whose disordered structure in water solution is more prone to adopt the most favourable conformation to adapt to elements of the allosteric site which are not essential for binding but are responsible for its functional consequences. This view point was corroborated by modelling presented below.

A second binding site for the CFTR Δ F peptide is present in the β subunit of CK2.

While a CK2 α binding site for CFTR peptides located at the interface between the α and the β subunits would account for the abrogation of the inhibitory efficacy of these peptides upon holoenzyme formation (see above) it is not sufficient to explain why the very same peptides display a stimulatory effect on CK2 holoenzyme when this is assayed with the majority of its established protein substrates [22]. A number of lines of experimental evidence support the view that such a stimulatory effect is mediated by a different CK2 region that is also able to bind the CFTR peptides, discretely located in the N-terminal domain of the β subunit. Firstly, it should be recalled that the β subunit is also able to interact with NBD1 in a manner which is enhanced by the F508 deletion [22]. Secondly the Δ F peptide is much more effective than the wild type peptide in preventing CK2 holoenzyme autophosphorylation which takes place at the N-terminal end of the β subunit (S2 and S3) and which is dependent on the integrity of an acidic cluster located somewhat down-stream in the same region [38]. Such an effect of the CFTR peptides (wild type and mutant) is illustrated in Fig. 4A using the phosphorylation of the beta subunit as a read out. Thirdly we have previously shown [22] that stimulation of CK2 holoenzyme by the CFTR Δ F peptide is especially evident with substrates belonging to class II, whose phosphorylation is usually prevented instead of being stimulated by the β subunit [17]. Interestingly such a down-regulation is mediated by the same acidic cluster which is required for autophosphorylation of the β subunit [39]. This point is exemplified in Fig. 4B by calmodulin, a typical class II substrate, whose phosphorylation by CK2 holoenzyme is almost negligible under basal conditions but it undergoes a 30-fold enhancement upon addition of 80 μ M Δ F peptide. Note however that if calmodulin is replaced by the eIF2 β (1-22) peptide, a class III substrate whose phosphorylation entirely relies on the β subunit, providing thereby its N-terminal segment structural elements important for substrate recognition, in this case [25], the effect of the CFTR Δ F peptide now becomes inhibitory (Fig. 4C). Thus three different signals could potentially be generated merely by the loss of one F residue. As in the case of downregulation of CK2 α , also the efficacy on CK2 holoenzyme is restricted to the CFTR peptides, whereas full length NBD1 Δ F has no appreciable effect (data not shown). The most likely explanation is the same discussed above, i. e. the greater rigidity of the NBD1 structure present, as compared to the derived peptide, hampering its adaptation to the responsive elements present on the kinase. We cannot of course exclude the possibility that the *in vivo* state of the NBD1 might be different and our purified preparation might not reflect that state (phosphorylation, ubiquitination etc. as described in [40]).

In summary, all available data obtained with CK2 holoenzyme can be explained assuming an interaction between the CFTR Δ F peptide and that very N-terminal region of the β subunit which is also responsible for autophosphorylation, down-regulation of class II substrate phosphorylation and recruitment of class III substrates. The more direct evidence that indeed this region of the β subunit mediates the stimulatory efficacy of the CFTR Δ F peptide was provided by experiments where polylysine was used to evoke calmodulin phosphorylation (Fig. 5). The binding site for polylysine as well as for other polybasic polypeptides, which, unlike polyamines, are able to activate CK2 holoenzyme toward calmodulin and other class II substrates, has been previously mapped to the region encompassing the 55-64 acidic cluster of the β subunit [41] suspected here to be also implicated in CFTR peptide binding. As shown in Fig. 5 polylysine exerts a dramatic stimulatory effect on calmodulin phosphorylation (lane 2), 20-fold more pronounced than that exerted by the CFTR Δ F peptide (lane 3). Interestingly if both polylysine and the CFTR Δ F peptide are present together, instead of observing an additive effect on stimulation, the phosphorylation drops 5-fold

(lane 4) with respect to that triggered by polylysine alone, reaching a value intermediate between those observed upon separate addition of each of the two effectors. This is precisely what one would expect assuming a competition of the two variably effective stimulators (polylysine and the CFTR Δ F peptide) for the same site, or at least their binding to two overlapping sites. Modelling further corroborates such a conclusion, as illustrated in the following section.

Molecular modelling.

To gain information about the possible modes of interaction between the CFTR peptides and CK2 the docking procedure described in the experimental section has been exploited.

We firstly focused on the interactions with the catalytic subunit which are responsible for allosteric down-regulation of its activity (see Fig. 2) and which are counteracted by the β subunit and by a β peptide encompassing residues 182-203 (Figs 3A and B). As shown in Fig. 6A the docking procedure maps the interacting region of α to its upper lobe where hydrophobic interactions take place between the Ile-Ile (506-607) doublet of the CFTR wild type peptide and a small superficial cleft of α formed by Ile-69, Leu-41 and Phe-54. Note that the same residues are also involved in the interactions with the full size β subunit and with the β peptide (PDB code 1DS5). In addition a π - π stacking occurs between peptide Tyr 515 and CK2 α Phe-54 just at the end of the Gly loop; this could lead to a weak alteration of the loop conformation resulting in slight inhibition of catalytic activity. The same hydrophobic interactions are maintained if the wild type peptide is replaced by the Δ F equivalent (Fig. 6B): in this case however also a couple of additional electrostatic interactions are observed, which could account for both tighter binding and more drastic inhibitory efficacy. In fact, owing to the F508 deletion, the C-terminal carboxyl of the peptide now moves back about 4Å, making an important electrostatic interaction with the side chain of Lys-44, while Arg-518 becomes electrostatically bonded to Glu-52. Both K44 and E52 participate to the Gly-loop and their interaction with the Δ F peptide is expected to hamper ATP binding to the loop, thus accounting for the observed increase in K_m for ATP (see Fig. 2B). Interestingly the deletion of Ile-507 fails to confer to the CFTR peptide the increased binding efficacy observed upon deletion of F508: as shown in Fig. 6C the remaining Ile occupies a position intermediate between those occupied by the two Ile residues (506 and 507) in both the wild type and the Δ F peptides. Consequently it is exposed toward the solvent, making contacts with two other residues of the peptide (V510 and Y515) leaving unoccupied the hydrophobic α cleft which otherwise provides an important anchorage to the peptide (compare Fig. 6C with 6A and 6B). The behaviour of the three CFTR peptides as highlighted by molecular docking also reflects in comparable outcome of protein-protein docking sampling (DS); this parameter is a percentage value of how much the preferred peptide pose is sampled with respect to the others. While with CFTR Δ I507 DS is only 1.5% (only 6 poses out of 400 total), with CFTR wild type and Δ F DS rises to 8.7% (35/400) and 23.7% (95/400) respectively.

We next moved to CK2 holoenzyme with an aim to map the site responsible for upregulation of its activity toward the majority of its substrates (with special reference to Class II subtype) and for inhibition of autophosphorylation. In agreement with the expectation based on biochemical data (Fig. 4A-C) the protein-protein docking strategy has localized this site on the N-terminal region of the β -subunit (see Fig. 6D). In this region a series of water exposed residues encompassing sequence between 33 to 61 define a particular surface characterized by a very small hydrophobic motif generated by F34, L36 and L39 and an extended highly charged platform including K33, R47, D50, E57, D59, E60, E61. All these residues display a striking complementarity with the CFTR peptide sequences. In particular the Δ F peptide uses its Ile-Ile 506-507 doublet to make a hydrophobic contact with β F34 and L36: this is followed by a strong electrostatic network involving D513 and E514 (bonded to K33 and R47, respectively) and R516/R518 interacting with E57 and E61. These interactions explain the complexity of the biochemical data shown in Fig. 4 (A-

C) since, remarkably, β residues engaged with the ΔF peptide are those previously demonstrated to be responsible for autophosphorylation, down-regulation of Class II substrates phosphorylation and recruitment of Class III substrates. Interestingly, and, once more in agreement with experimental data, the interactions with β are partially lost if the ΔF peptide is replaced by the wild type one: insertion of F508 in fact modifies the electrostatic network down-stream causing the failure of E514 and R518 to interact with K33 and with E61, respectively. Also consistent with the modelling is the loss of stimulatory activity caused by the deletion of the C-terminal doublet (Y517-R518).

DISCUSSION

The most disconcerting feature of CK2 probably comprises its lack of stringent regulation. Although CK2 holoenzyme is endowed with two non-catalytic β subunits, these don't seem to act as *sensu stricto* regulatory elements since they are not required to either turn on (as cyclins do with CDKs) or turn off (as R subunits do with PKA) the catalytic subunits. Furthermore, as pointed out by Raaf et al. [18] all the numerous crystal structures of these latter solved thus far, either alone or in combination with the β subunits and with a variety of ligands, are invariably in the active conformation.

The β subunits, on the other hand, besides providing a docking platform for interacting proteins and modulators [42] also affect the stability of the kinase and, more importantly, control its targeting of protein substrates, which, accordingly, have been assigned to three classes [18]: class I includes the majority of substrates, whose phosphorylation is efficiently performed irrespective of the presence of β ; class II is composed of substrates, exemplified by calmodulin, whose phosphorylation is drastically inhibited upon formation of the holoenzyme; while substrates belonging to class III are those, like eIF2 β , whose phosphorylation is conversely crucially dependent on the β subunit, being readily performed by the holoenzyme, but never by the isolated catalytic subunits. It has been proposed that these very different behaviours are at least partially accounted for on one hand by the potential of the β -subunit to act as a pseudo-substrate inhibitor by virtue of its highly acidic N terminal segment also harbouring an autophosphorylation site at Ser-2 and 3 [38, 43], yet on the other by the requirement of the β subunit to generate the binding site for a limited number of substrates which are not effectively recognized by the catalytic subunit alone [44, 25].

Whether in vivo the formation of CK2 holoenzyme is a reversible reaction or not, is still an open question [7]. On one hand, the very high affinity binding between catalytic and non-catalytic subunits ($K_D = 40$ nM) [22] argues against dissociation of the holoenzyme under physiological conditions; on the other it has been recently shown that an exchange between assembled and "free" subunits can take place, albeit very slowly [45]. In any case the existence in cells of CK2 catalytic subunits not combined with the β subunits has been reported from time to time (e.g. [46-48]).

Especially pertinent to the outcome of our work is the striking abundance of CK2 α not accompanied by a concomitant rise in CK2 β observed in CEM drug resistant cells (R-CEM) as compared to CEM drug sensitive (S-CEM) cells [49] given that in this case the elevated CK2 α was not paralleled by any increase in catalytic activity measured with the specific peptide substrate. This suggests that in R-CEM cells CK2 α is in an inactive form.

Interestingly it has been recently shown that a flexible region of CK2 α exists at the interface with the β -subunit [20], which could be induced to adopt an inactive conformation, should there be a ligand able to bind to it instead of to β [18].

This region overlaps fairly well the allosteric site where, based on the biochemical data and molecular docking analysis presented here, we propose that the binding of the CFTR- $\Delta F508$ peptide takes place, inducing or possibly stabilizing an inactive conformation of the catalytic subunit (see Fig. 6). According to our modelling, inactivation is mediated by a displacement of the Gly-loop due to interactions with the C-terminal residues of the ΔF peptide (see Fig. 6 and comments in the text).

These interactions are much weaker with the wild type CFTR peptide and with the $\Delta I507$ equivalent, thus accounting for failure of these peptides to inhibit CK2 α as drastically as the ΔF peptide (see Fig. 1)

Collectively taken our data are consistent with the scenario depicted in Fig. 7, where in the presence of CFTR fragments bearing the F508 deletion, newly synthesized CK2 α is sequestered in an inactive complex less prone to readily interact with the β subunits. There is abundant evidence that CFTR degrades in the proteasome when F508 is deleted but the same fate is reported to a lesser degree for wild type CFTR as well [40].

To note that in this manner the CFTR ΔF fragments not only promote allosteric inhibition of “free” CK2 α , immediately resulting in down-regulation of class II substrates, but will also induce, by subtracting α from the $2\alpha + \beta_2 \rightleftharpoons \alpha_2\beta_2$ equilibrium, a gradual decrease of CK2 holoenzyme, expected to reduce the phosphorylation of the remaining targets of CK2 as well. This latter effect however could be overcome, or at least attenuated, by the binding of the CFTR ΔF peptide to its second allosteric site highlighted in this work, located at the N-terminus of CK2 β , whose occupancy results in up-regulation of CK2 holoenzyme toward all its substrates [22] with the exception of those belonging to class III (e.g. the eIF2 β peptide, see Fig.4C). Thus CK2 activity could be fine tuned.

Assuming that a mechanism like that schematically depicted in Fig. 7 is operating in R-CEM cells (regardless of the nature of the allosteric effector responsible therein, see [50] for possible candidates), this could account for the puzzling observation that in those cells, despite the fact that the increase of CK2 α was not accompanied by any increase of activity toward the specific peptide substrate, nevertheless the phosphorylation of some endogenous targets of CK2 was significantly enhanced [49].

Our present data may also provide a breakthrough toward the understanding of some enigmatic aspects of CF pathology, which are difficult to reconcile with the loss of function of CFTR, notably alterations in the inflammatory responses and a six fold excess of bowel cancer in young patients some of whom are in teenage [51]. Considering the pleiotropy of CK2 and its implication in pathological events like inflammation, infections, and tumorigenesis [15], the hypothesis that some of the derangements observed in CF pathology are caused by CK2 through the generation of biologically active CFTR peptides, will deserve attention in future experiments given that CFTR is rapidly degraded by the proteasome which in turn predicts 8-12mer peptides of varying composition based on its known catalytic properties. Pertinent to this may be the results obtained with peptides including $\Delta I507$ mutation. This deletion, otherwise very similar to the $\Delta F508$ one as far as its consequences on the stability of CFTR is concerned [28], nevertheless sharply differentiates in its population prevalence which is negligible compared to $\Delta F508$ [29]. This observation suggests that the $\Delta I507$ mutation is unable to provide the selective advantage of the $\Delta F508$ mutation (note this only pertains to heterozygotes) although both promote the same loss of function of CFTR in a CF disease context where loss of ion channel function plays a critical role. Our data is consistent with the hypothesis that the selective advantage of the ΔF mutation in heterozygotes is due to a gain, rather than a loss of function. Furthermore, that deregulation of CK2 mediates this effect is corroborated by our data showing that the $\Delta I507$ peptide is unable to exert the same efficacy of the $\Delta F508$ peptide on CK2 activity.

We are aware that for the time being a serious limit to our working hypothesis arises from paucity of information about the endogenous generation and actual concentration in CF cells of CFTR ΔF fragments of the right size and composition, that might equate to our in vitro work with their synthetic derivatives. Yet it is now almost axiomatic that around two thirds of newly synthesised wild type CFTR is proteasomally degraded, a figure which rises to over 90% for the F-deleted mutant [40]. Interestingly this apparently wanton destruction does not occur with other ABC proteins such as P glycoprotein which is efficiently processed without degradation suggesting

CFTR is somehow a special case. For example, CFTR is even targeted for destruction through ubiquitination prior to its complete synthesis on the ribosome [40].

Evidence is now available that the same CFTR peptides used in this in vitro study, when expressed by viral infection in a foetal animal model do display biological effects, notably enhancement of Wnt reporter gene expression, which may well be mediated by CK2 [23]. Other caveats arise from the extremely variable dynamics of CFTR expression (and degradation), with peaks of 50-fold more mRNA found in foetal life compared to adult lung [52], leaving open the possibility that under special circumstances the proposed degraded peptide concentration might be higher than expected. We must also consider the crucial importance that the conformation of such peptides appears to have in order to display biological activity toward CK2. This latter point is highlighted by the failure of the very same 500-518 sequence which in the peptide is quite effective, to display any similar efficacy on both CK2 α and CK2 holoenzyme once it is incorporated into the whole NBD1 domain and by a number of unpublished observations undertaken in our labs showing that by just changing the method of solubilization of the peptides (e.g. DMSO vs water) their efficacy on CK2 sometimes is dramatically impaired.

In his wide ranging review [40], one of the founding fathers of the CFTR field, J. Riordan, recently commented that it remains controversial as to whether CF really is a pleiotropic disease. It is clear that strong views are held on either side of the debate (CFTR as a channelopathy, CFTR in the innate immune system etc). Our aim is to provide a testable route to measure the degree of CF pleiotropy and our future efforts will be directed to assess the presence of these peptides and to monitor corresponding alterations in CK2 activity and targeting in cells where these peptides may well be generated. Regardless to the outcome of these future studies it should be underlined that our present data substantially contribute to the definition of allosteric sites where cellular ligands other than CFTR peptides may also bind to modulate CK2 activity. Pertinent to this may be the intriguing observation that the sequence surrounding the F508 deletion in CFTR is fairly well conserved in a number of proteins (unpublished observation) with special reference to some histones which stimulate CK2 holoenzyme by binding to the N-terminal region of the β subunit [41]. It should be highlighted in particular that histone H4, which has been shown to be a powerful stimulator of CK2 holoenzyme [41], shares remarkable similarity with the CFTR ΔF peptide (Table S2 of Supplementary Material), whereas this is not the case with respect to histone H1 whose efficacy on CK2 holoenzyme activity was found to be negligible [41]. This discloses the possibility that a number of protein and peptide ligands sharing the motif found around the deleted F508 of CFTR could function as modulators of CK2 activity.

Acknowledgments

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Figure Legends

Fig. 1: Sequence-determined variability of inhibition of CK2 α by short CFTR peptides straddling the F508 region of wild type or mutant CFTR.

The percentage change in CK2 α activity (ordinate, relative to no-peptide control as 100%) was determined against the specific synthetic peptide R₃AD₂SD₅ as phosphorylatable substrate. A bi-directional effect on CK2 α occurs between F508 and negative charge versus hydrophobicity at position 510. For this and subsequent figures, CK2 α activity was determined as described in the Experimental Section and all data points are calculated from the mean of at least three independent experiments with SEM never exceeding 15% (not shown for clarity).

Fig. 2: Kinetic analysis of the mode of inhibition of CK2 α activity towards substrates induced by the CFTR Δ F508 peptide.

In A the double reciprocal plot of the phosphorylation of increasing concentrations of the CK2 α substrate calmodulin either in the absence (squares) or in the presence of 160 μ M peptide (triangles) reveals no change in K_m (abscissa intercept) but a near three fold decline in V_{max} (ordinate intercept) induced by the peptide consistent with a non-competitive action.

In B, as ATP concentrations rise the double reciprocal plots reveal two phases. Note the abrupt increase in the slope of the double reciprocal plot induced by sub-maximal concentrations of peptide as reflected in alterations in both V_{max} and K_m .

Fig. 3: CK2 α inhibition by the CFTR Δ F peptide is counteracted by the β subunit and by NBD1 Δ F508.

CK2 α activity was determined, as described in the Experimental Section, against the specific synthetic peptide R₃AD₂SD₅ as phosphorylatable substrate. In A the dose-dependent inhibitory power of the peptide Δ F was evaluated either against CK2 α alone or on CK2 α preincubated with a 10-fold molar excess of CK2 β to create the holoenzyme. Note the loss of peptide-induced inhibition when the holoenzyme is present. In B the ability of the β -peptide 181-203 to stimulate the activity of CK2 α was evaluated either in the absence (circles) or in the presence of 40 μ M Δ F peptide (triangles). Note that the degree of inhibition of CK2 α induced by the Δ F peptide (compare zero values on abscissa) is relieved as [β -peptide] increases. The percent change of the resulting decrease of inhibition is plotted in C. In D the inhibitory effect of increasing concentrations of the Δ F508 peptide on CK2 α activity was determined either in the absence or in the presence of recombinant NBD1 Δ F508 full length protein (50 μ M). Controls with Δ F NBD1 alone (in the absence of the peptide substrate) were run, whose phosphorylation was < 5% of that of the peptide, and subtracted from this. Controls of CK2 α activity tested either in the absence or in the presence of NBD1 Δ F508 revealed no significant effect i.e. the 100% absolute values were similar (not shown). Thus the inhibitory effect of the short peptide towards CK2 α was dramatically reduced by the full length protein domain that by itself had no effect on the kinase activity.

Fig. 4: Variable effects of the interaction of CK2 holoenzyme with the wild type and Δ F508 CFTR-derived peptides.

Autophosphorylation of CK2 β subunit and assays on holoenzyme activity towards substrates (peptide and protein) were performed as described in the Experimental Section.

A: Inhibition of the CK2 β -subunit autophosphorylation by increasing concentrations of either wild type or Δ F508 CFTR peptides.

B: stimulation of the phosphorylation of calmodulin induced by increasing concentrations of either wild type or Δ F508 CFTR peptides (contrast V_{max} decline in Fig 2A). Note the enhanced relative

potency of the F508-deleted peptide towards calmodulin phosphorylation (compare open bars) when holoenzyme is present against its contrasting effects on CK2 α alone.

C: Yet the very same peptide has inhibitory actions towards the phosphorylation of the eIF2 β -derived peptide as the concentrations of this Δ F508 CFTR peptide rise. Note no effect when eIF2 β -derived peptide (class III substrate) is replaced by another peptide (class I substrate) showing selectivity of action towards different substrates.

Figure 5. The stimulatory efficacy of polylysine on CK2 holoenzyme is reduced by the CFTR Δ F peptide. Calmodulin (CaM) underwent phosphorylation by CK2 holoenzyme in the absence (lane 1) or presence of either 330 nM polylysine (PL) or 80 μ M CFTR Δ F peptide (lanes 2 and 3, respectively), or both (lane 4). The stimulatory effect of polylysine observed in lane 2 was attenuated by the CFTR Δ F peptide (lane 4), which by itself is capable of enhancing phosphorylation of CaM (lane 3), albeit much less efficiently than polylysine. These results argue for a competition between polylysine and the CFTR Δ F peptide for the same anchoring site.

Fig. 6: Molecular docking of wild type and mutated CFTR peptides by CK2 α and by CK2 holoenzyme.

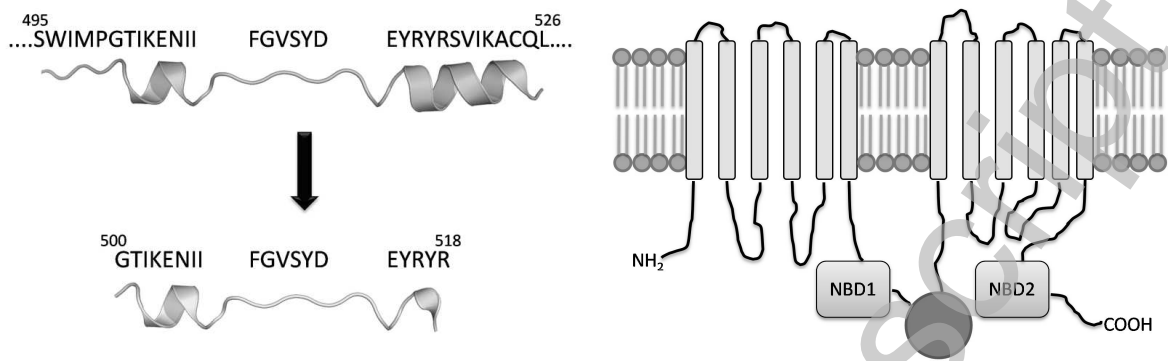
In A, B and C the main molecular contacts between CK2 α (green), CFTR peptides (red), CK2 β (blue; D only) are shown as complexes between CK2 α subunit and CFTR peptides wild type, Δ F508 and Δ I507. Using Glu52/Ile69 (green) as a CK2 α fixed reference points note the different contacts for Phe54 and Leu41 (both green) against differently folded CFTR peptides in A,B, C. In D the interactions between the β -subunit of CK2 holoenzyme and the Δ F508 peptide are highlighted. Dotted red areas denote hydrophobic interactions, blue dotted lines indicate salt bridges. Docking sampling (DS) parameter for each complex, calculated as described in the Experimental Section and discussed in the text, is also reported.

Fig. 7: Schematic illustration of the proposed dual effect of CFTR fragments on CK2 α and on CK2 holoenzyme.

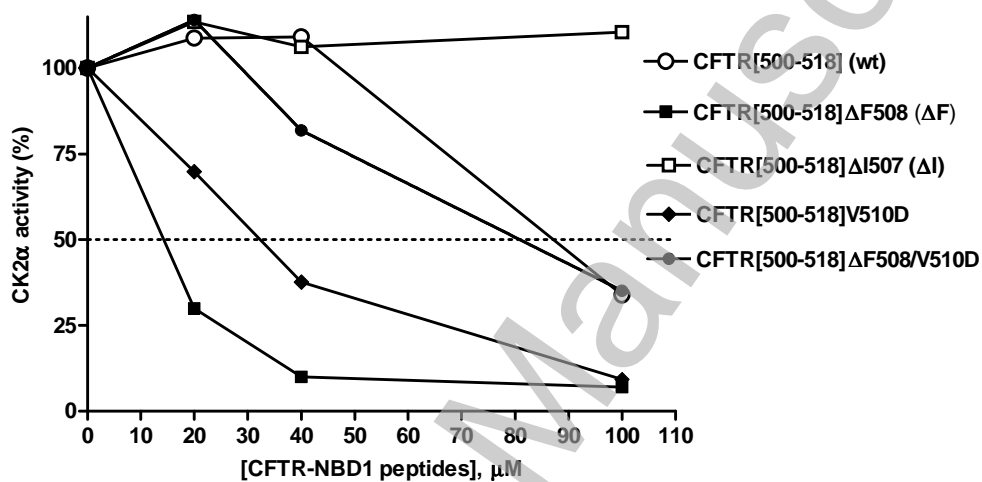
The binding of Δ F peptides to CK2 α subunit allosteric site disclosed by Figure 2 locks CK2 α in an inactive conformation and hampers the formation of the active holoenzyme (new steady state based on competition between CFTR peptides and CK2 β outlined in Figure 3). The effect of binding of Δ F peptides to the β subunit within the CK2 holoenzyme is variable depending on the class of substrate affected (see Figure 4) but with most substrates is expected to induce activation [22]. In this model, as concentrations of the CFTR peptide with F508 deleted rise, calmodulin and more in general class II substrates would be hyper-phosphorylated whereas phosphorylation of eIF2 β and other class III targets would be inhibited.

Table 1. IC₅₀ values for the inhibition of CK2 α by CFTR-derived peptides.

The parent peptide (1, wt) reproduces the 500-518 sequence of human CFTR. It belongs to the NBD1 domain of CFTR schematically represented in the right cartoon (in practice, the two NBDs are thought to create a dimeric sandwich trapping two nucleotides). Between brackets in the table are the simplified acronyms used to indicate the background of the peptides that are reported.



N.	Peptide	IC ₅₀ (μ M)
1	GTIKENIIFGVSYDEYR (w.t.)	85
2	IIFGVSYDEYR	99
3	GVSYDEYR	>250
4	GTIKENIIFG	241
5	IIFGVSYDE	>250
6	GTIKENIIFGVSYDE	>250
7	GTIKENII-GVSYDEYR (Δ F)	15
8	GTIKENI-FGVSYDEYR (Δ I)	>250
9	GTIKENIIFGDSYDEYR (V510D)	33
10	GTIKENII-GDSYDEYR (Δ FV510D)	81
11	II-GVSYDEYR	23
12	II-GVSYDEYR	>250



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Figure 1

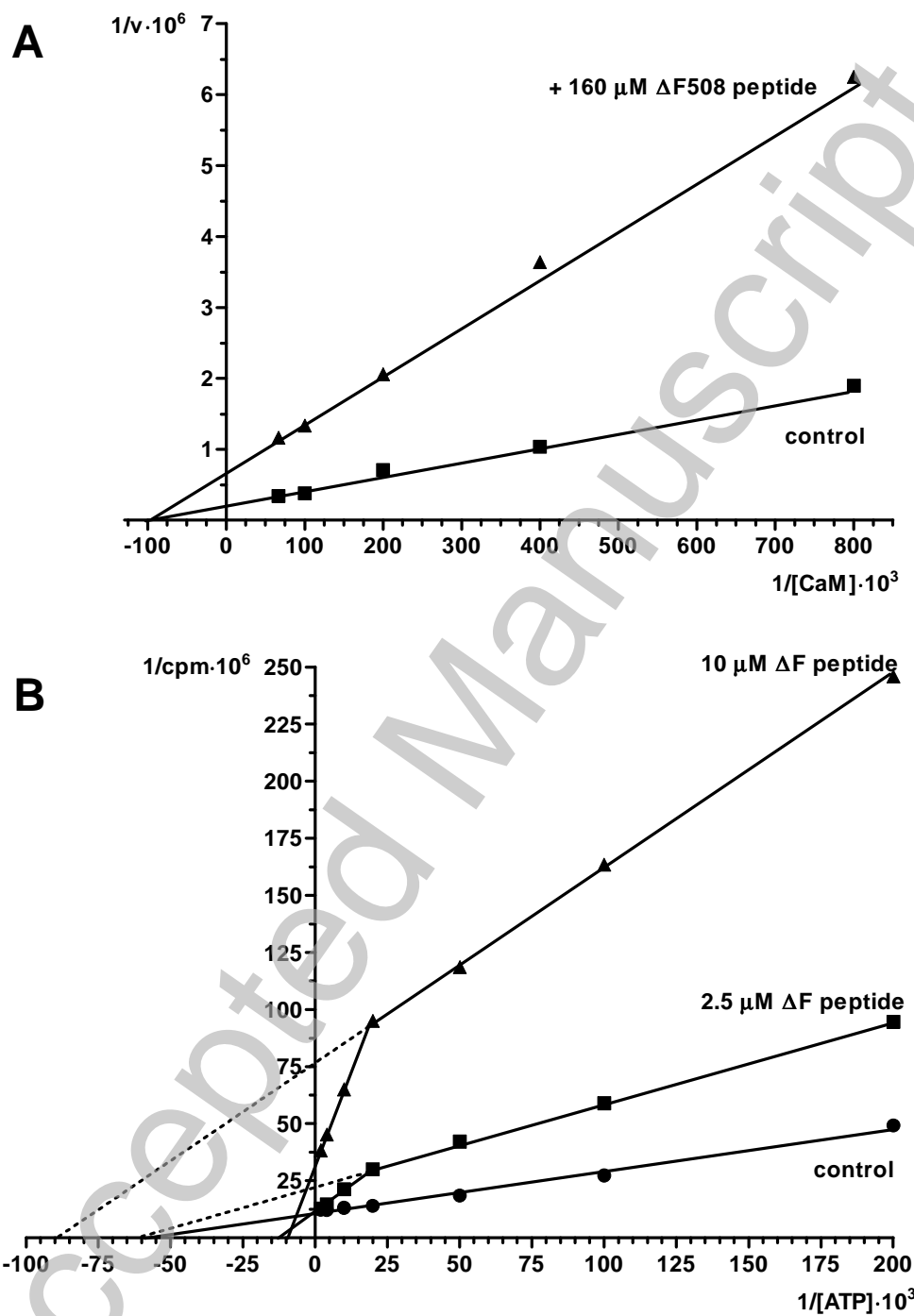


Figure 2

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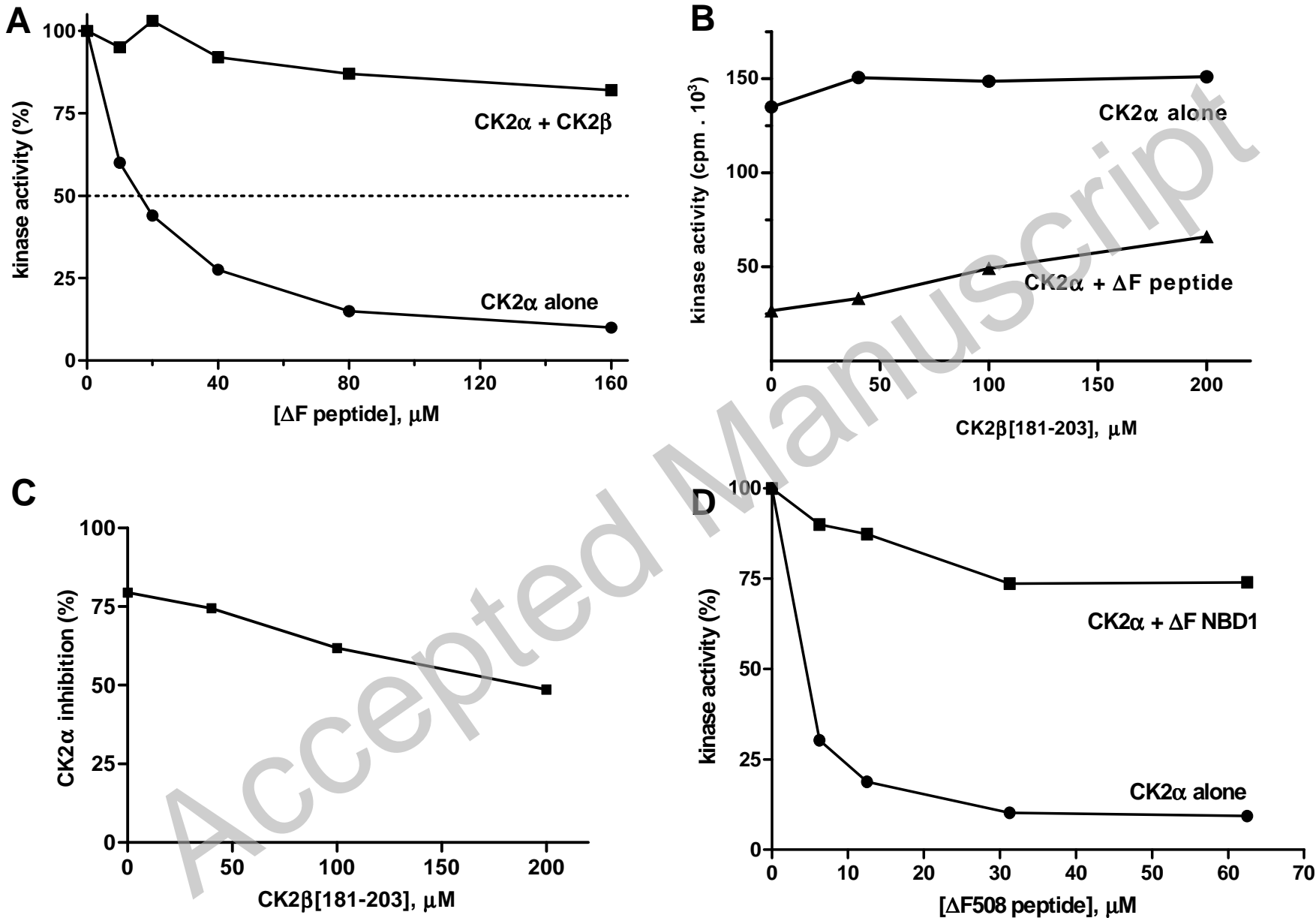
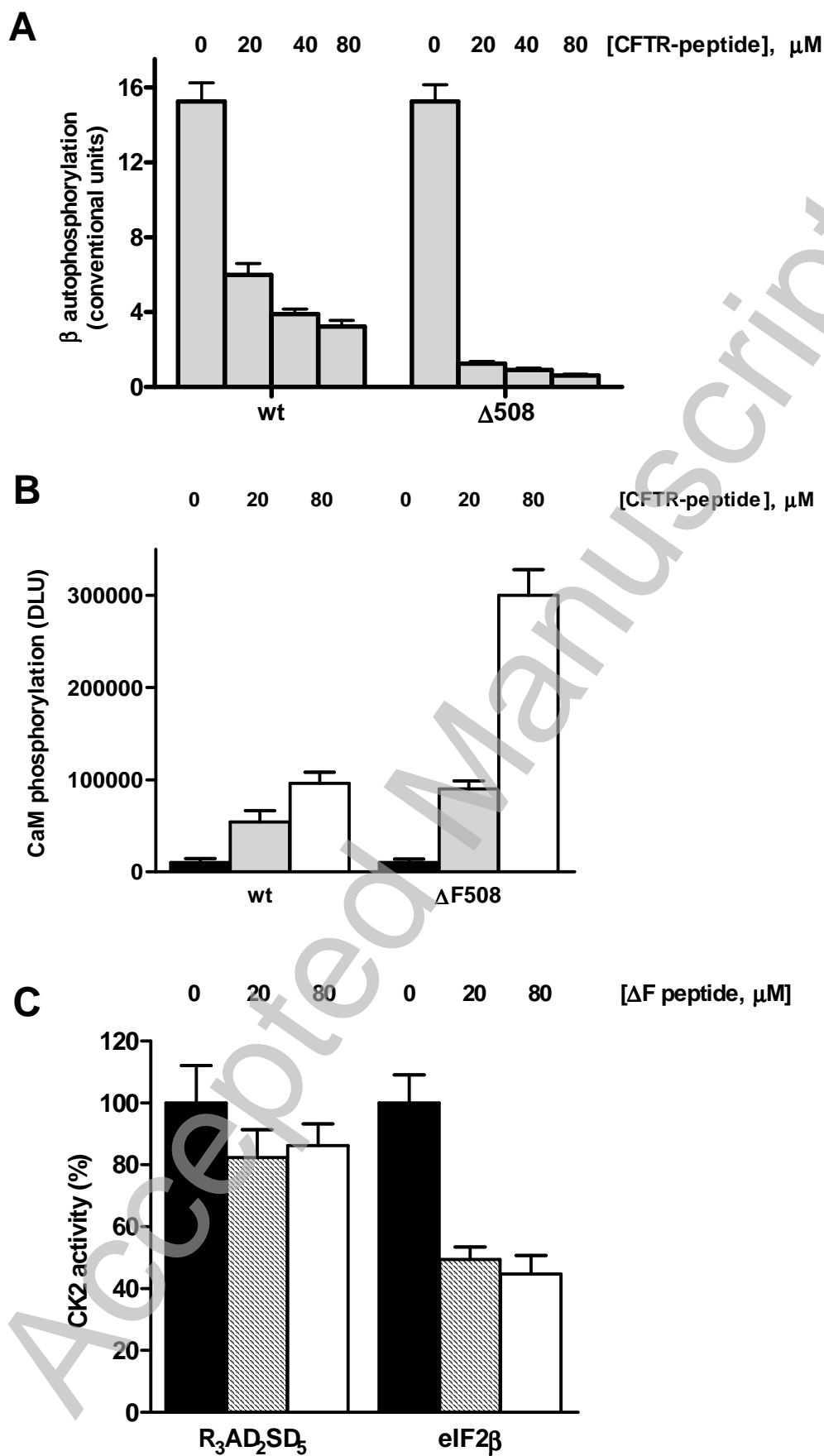


Figure 3



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Figure 4

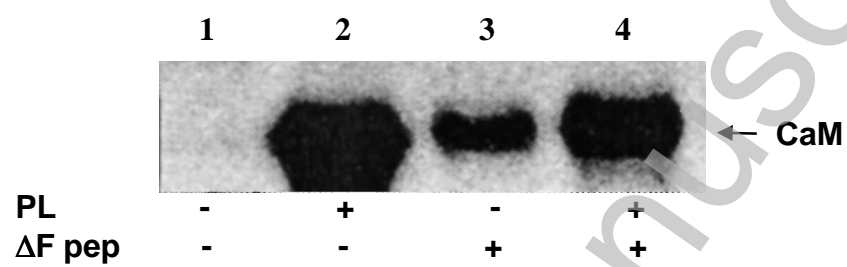


Figure 5

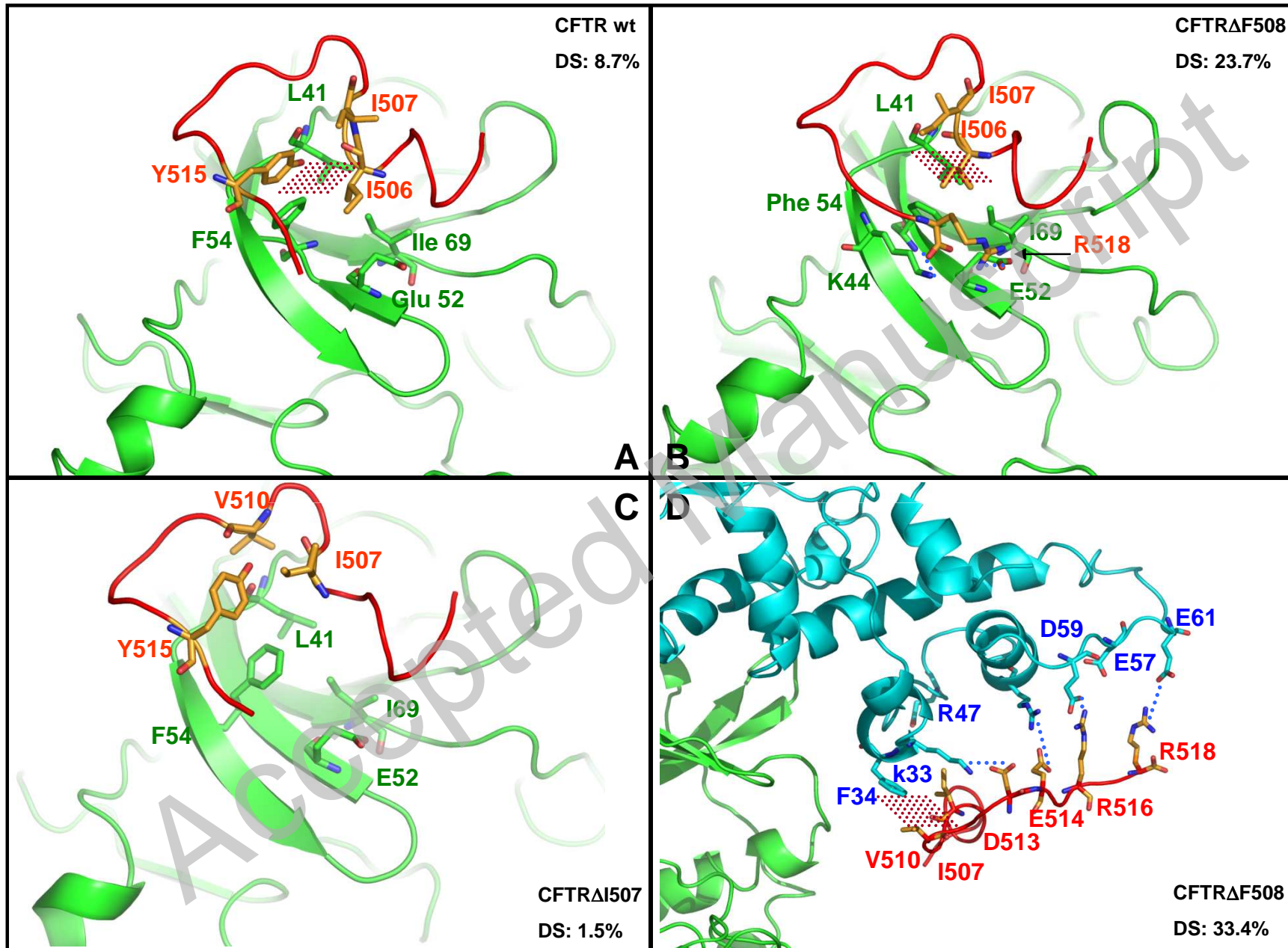


Figure 6

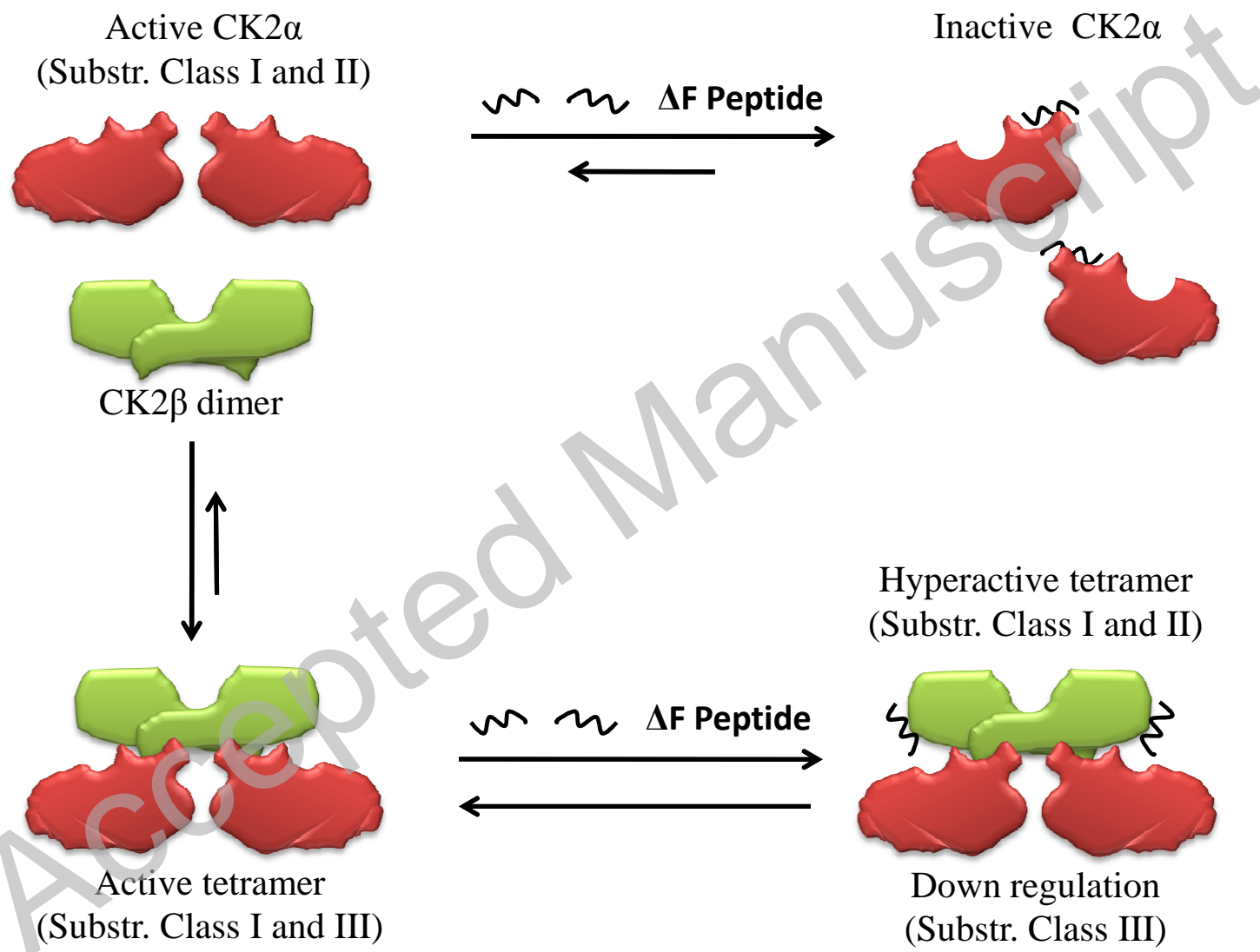


Figure 7