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The Regulatory β Subunit of Protein Kinase CK2 Contributes to the Recognition of the Substrate Consensus Sequence. A Study with an eIF2β-Derived Peptide[†]

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ABSTRACT: CK2 is a ubiquitous and pleiotropic Ser/Thr-specific protein kinase that phosphorylates more than 300 protein substrates at sites specified by an acidic consensus sequence in which positions $n + 3$ and $n + 1$ are particularly important. Recognition of substrates by CK2 is known to rely on basic residues located in the catalytic site of the α subunit which make electrostatic contacts with the negative charges in the substrate consensus sequence, thereby assuring optimal binding; the regulatory β subunit is believed to play a protective and stabilizing role. We describe a biochemical and structural analysis of CK2-mediated phosphorylation of a 22-mer synthetic peptide corresponding to the N-terminal tail of the eukaryotic translation initiation factor eIF2β. Results demonstrate that this peptide still displays phosphorylation features similar to full-length eIF2β and the CK2 β subunit also contributes to recognition of the protein substrate by establishing both polar and hydrophobic interactions with specificity determinants located downstream from the phosphoacceptor site. In particular, the N-terminal domain of the β subunit appears to be of crucial importance for optimizing high-affinity phosphorylation of the eIF2β peptide. This domain includes an acidic cluster whose electrostatic contacts with basic residues of the substrate attenuate intrasteric pseudosubstrate inhibition while strengthening substrate−kinase binding.

Protein kinase CK2[1] (also known as casein kinase 2 or II) is a multifunctional and pleiotropic Ser/Thr-specific protein kinase that is essential for viability and involved in cell growth, proliferation, and survival (reviewed in refs *1–5*). Mounting evidence supports the view that alterations in CK2 activity can have profound effects in the context of transformation and cancer, although the molecular basis for these effects still remains incompletely understood (*4, 5*). The structure of CK2 consists of a heterotetrameric complex of α, α′, and β subunits in which two catalytic subunits are linked via a dimer of regulatory β subunits to form configurations such as $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$, depending upon the cell type. Biochemical and crystallographic data suggest that the enzyme forms supramolecular complexes of various sizes and shapes (*6–9*) which represent the true active form of the kinase at physiological salt concentrations. However, the mechanism(s) and effector(s) potentially regulating the association/dissociation of these complexes remain to be characterized. This model does not exclude the possibility that free catalytic and regulatory subunits might in certain situations play an important role in the specific targeting of cellular partners (*3*).

Studies based on site-directed mutagenesis (*10, 11*) demonstrated that the substrate's acidic consensus sequence (*9, 10*) is recognized by a stretch of lysines spanning residues 74−80 of helix C of the catalytic subunit together with a number of other basic residues located nearby in the active site, the combination of which forms a network of positive charges responsible for the correct binding and orientation of the phosphorylatable substrate. These studies led to the conclusion that only the catalytic site of the α subunit is responsible for recognition of the local determinants surrounding the phosphorylatable serine or threonine of the protein substrate. Studies of the β subunit (*12*) demonstrated that it stabilizes the catalytic subunit against denaturing agents, resulting in a general increase in kinase activity toward most substrates. The C-terminal domain of the regulatory subunit was found to be particularly important for this stimulatory effect (*13, 14*). Subsequent resolution of the 3D structure of the CK2 holoenzyme revealed that this domain stabilizes molecular contacts with the other regulatory subunit and with one of the two catalytic subunits of the heterotetramer, mainly through hydrophobic interactions (*15*). The stimulatory property of the β subunit usually predominates over a parallel negative effect conferred by an acidic domain located near its N-terminus (residues 55−70) that acts as an intrasteric pseudosubstrate (*16*). Several lines of evidence, including the fact that CK2 is stimulated by polybasic compounds, point to the conclusion that this region of the β subunit does

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[1] Abbreviations: CK2, casein kinase 2; eIF2β, eukaryotic translation initiation factor 2 β subunit.

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indeed downregulate CK2 activity. It is important to note that the electrostatic contacts between the β subunit's N-terminal acidic domain and basic residues in the active site of the α subunit are made possible by higher order interactions between distinct CK2 tetramers rather than within a single holoenzyme (*8, 9, 15*). In fact, this acidic stretch is located a considerable distance away from the active site of either catalytic subunit of the heterotetramer, thus ruling out the possibility of electrostatic "intratetramer" interactions.

Besides enhancing the stability and catalytic activity of CK2, the regulatory β subunit has also been found to cooperate in modulating substrate selectivity. This has been demonstrated for many CK2 cellular partners including, among others, Nopp140 (*17*), DNA topoisomerase II (*18*), p53 (*19*), FAF-1 (*20*), and FGF-2 (*21*). Rather than involving a simple mechanism of consensus sequence recognition, phosphorylation of these proteins by CK2 is believed to rely on formation of a molecular platform by the β subunit which allows docking and/or recruitment of the substrate or potential regulators.

Among the CK2 cellular partners interacting with the regulatory β subunit, HIV-1 Rev and calmodulin, both of which are confirmed in vivo substrates for CK2, have been found to display unique and contrasting features. In particular, while phosphorylation of HIV-1 Rev is dramatically stimulated by the presence of the β subunit, phosphorylation of calmodulin is strongly inhibited by it (*22, 23*), with the β subunit's N-terminal acidic domain playing a crucial role in both cases. HIV-1 Rev protein is in fact phosphorylated exclusively by the oligomeric CK2 holoenzyme (*22*), as a result of a dual interaction with both electrostatic and hydrophobic domains of the β subunit. On the contrary, while calmodulin is a substrate for the free α subunit, its phosphorylation by the heterotetrameric CK2 holoenzyme requires the addition of polybasic compounds (*23*). This latter property involves the N-terminal acidic domain of the β subunit, whose electrostatic interaction with the positively charged chains of the polybasic effectors allows the CK2 catalytic site to come into a tighter molecular contact with the central helical region of calmodulin, which would otherwise be hindered by the presence of the β subunit.

This report describes the contribution of the β subunit to phosphorylation of eukaryotic translation initiation factor 2 (eIF2β), another atypical substrate of CK2 whose phosphorylation, mainly occurring at the N-terminal Ser2 (*24*), was recently found to require the holoenzyme form of the kinase (*25*), as described above for HIV-1 Rev. However, in contrast to HIV-1 Rev, whose requirement for the CK2 holoenzyme is lost upon removal of sequences surrounding the phosphorylation site, a 22-mer peptide derived from eIF2β remains fully dependent on the β subunit for its phosphorylation (*26*). Analysis of a panel of substituted/truncated derivatives of this peptide as well as the use of a number of mutants of full-length eIF2β verified that the CK2 regulatory subunit cooperates with the catalytic subunit in the recognition of local specificity determinants on the substrate.

EXPERIMENTAL PROCEDURES

Kinases. Native CK2 (nCK2) was purified from rat liver (*27*). Human recombinant α and β subunits of CK2, either wild type or mutated, were expressed in *Escherichia coli*, and the holoenzyme was reconstituted and purified as previously described (*10*).

Peptide Synthesis. The peptide eIF2β[1−22] and its derivatives were synthesized by the solid-phase peptide synthesis method using an automatized peptide synthesizer (model 431-A; Applied Biosystems, Foster City, CA). The fluoren-9-yl-methoxycarbonyl (Fmoc) strategy (*28*) was used throughout the peptide chain assembly, with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) serving as coupling reagents. HMP resin (Applied Biosystems) and Fmoc-Pro-NovaSyn TGT resin (Novabiochem, Bad Soden, Germany) were employed as solid supports. The side-chain-protected amino acid building blocks used were Fmoc-Glu(*tert*-butyl), Fmoc-Asp(*tert*-butyl), Fmoc-Ser(*tert*-butyl), Fmoc-Thr(*tert*-butyl), and Fmoc-Lys(*tert*-butyloxycarbonyl). Peptides were cleaved from the support resins by incubation with 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 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.

Plasmids, Protein Expression, and Purification. The pET16b plasmid with the insert encoding full-length wild-type human eIF2β was a gift from Drs. Beullens and Bollen (Department of Molecular Cell Biology, KUL, Leuven, Belgium). Amino acid substitutions were made by polymerase chain reaction using the QuikChange II site-directed mutagenesis kit (Stratagene), with the appropriate primers and templates. In all cases, mutations were confirmed by DNA sequencing. The His_6-tagged fusion proteins encoded by these vectors were expressed in *E. coli* BL21(DE3)pLysS cells (Invitrogen) and purified by Ni^{2+}-Sepharose high-performance (GE Healthcare) chromatography.

CD Analysis. CD spectra were recorded with a Jasco (Easton, MD) spectropolarimeter. The instrument was calibrated with *d*-(+)-10-camphorsulfonic acid. Far-UV CD spectra were recorded at 25 °C at a peptide concentration of 30 μM in phosphate-buffered saline (pH 7.4) (Sigma-Aldrich, Germany; Cod. P3813) using 0.1 cm path length quartz cells. The mean residue ellipticity [Θ] ($deg \cdot cm^2 \cdot dmol^{-1}$) was calculated from the formula $[\Theta]_{MRW} = (\Theta_{obs}/10)MRW/lc$), where Θ_{obs} is the observed ellipticity at a given wavelength, MRW is the mean residue weight of the peptide(s), l is the cuvette path length in cm, and c is the peptide concentration in g/mL.

Protein−Protein Docking. Protein−protein docking is a computational tool useful for predicting the three-dimensional structure of protein complexes from the coordinates of its subunits. Most protein−protein docking algorithms use a rigid docking approach, in which both the backbone and side chains of component structures are frozen.

To correct for the absence of flexibility, a two-stage methodology was adopted. In the first stage, the two monomers were treated as rigid bodies, and all of the rotational and translational degrees of freedom were fully explored. In the second stage, a small number of structures

derived from the first step were refined in the docking zone using side-chain rotamers and energy minimization strategy (*29*).

We first performed docking analyses with four protein−protein docking approaches, namely, Zdock (*30*), Gramm (*31*), Bigger (*32*), and Escher (*33*). We performed one docking run for each approach, with 1000 solutions obtained for each. We considered only the best 10% structures ranked from the four analyses, and after comparing them, we selected one or more conformation(s) of the complex to be refined in the second stage. In the second phase of our approach, a systematic conformational search was used to generate side-chain conformations of amino acids with steric clashes, using the MOE (*34*) rotamer explorer methodology. The best solution suggested during this phase, according to the available crystal structure interaction data, was selected for further analysis. All interaction zones were considered and the nearest local free energy minimum was determined by using the AMBER99 (*35*) force field and a gradient-based minimization.

Phosphorylation Assays. The CK2 assay has been described in detail previously (*36*). Phosphorylation reactions (25 μL) contained CK2 α subunit (0.5 pmol) or α plus β subunits (0.5 pmol each), peptide (0.2 mM unless otherwise indicated) or protein substrates, 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, and 100 μM [γ-^{32}P]ATP (specific radioactivity 500−1000 cpm/pmol); NaCl was omitted from the experiments utilizing the effector polylysine (330 nM). The reactions were incubated for 10 min at 37 °C and stopped by cooling in ice and absorption on phosphocellulose paper. Papers were washed three times with 75 mM phosphoric acid, dried, and counted in a scintillation counter. The phosphate incorporated into eIF2β-derived peptides devoid of basic residues and not suitable for binding to phosphocellulose filters was determined after conversion of [γ-^{32}P]ATP into P_i and phosphomolybdic complex extraction as described previously (*37*). In vitro phosphorylation of His_6-tagged eIF2β forms was performed at 30 °C for 10 min in a 50 μL volume of the reaction mixture described above for the synthetic peptides. Reactions were stopped by adding Laemmli loading buffer and boiling followed by SDS−PAGE and Coomassie staining. Radiolabeled bands were detected in a PhosphorImager and quantified by densitometry.

Determination of Phosphorylation Kinetics. Initial velocities were determined at each of the substrate concentrations tested. K_m values were calculated from Lineweaver−Burk double reciprocal plots of the data.

RESULTS

Full-Length eIF2β and Its N-Terminal Segment 1−22 Are Phosphorylated by Protein Kinase CK2 with Comparable Features. Eukaryotic translation initiation factor eIF2β is phosphorylated by protein kinase CK2 at Ser2 and, to a lesser extent, at Ser67 (*24*). Comparable kinetics of phosphorylation were reported for a C-terminal truncated form which, similar to the full-length protein, was quite efficiently phosphorylated by CK2 heterotetramers but not by the free catalytic subunit (*25*). Such behavior would identify eIF2β as a class III CK2 substrate, which are distinguished by appreciable phosphorylation only in the presence of the regulatory subunit (*1*).

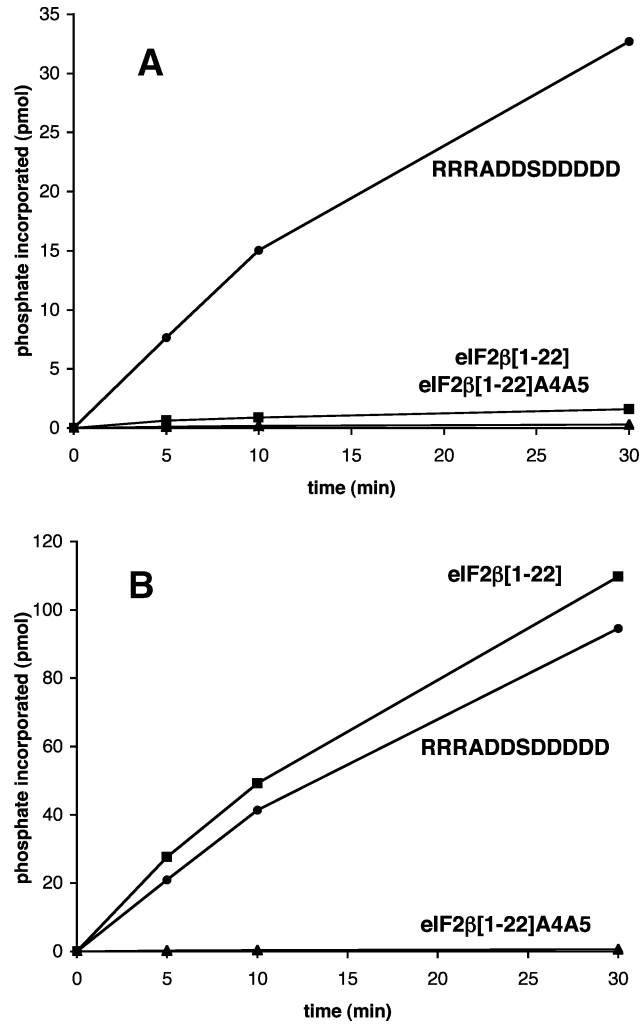

FIGURE 1: Time courses of phosphorylation of the eIF2β[1−22] peptide by CK2 α subunit and holoenzyme. The phosphorylation reaction was performed and evaluated as described in the Experimental Procedures using eIF2β[1−22] peptide (100 μM) and either free α catalytic subunit (0.5 pmol, panel A) or an equimolar mixture of the α and β subunits to reconstitute in vitro the heterotetrameric holoenzyme (panel B).

Ser2 is followed on the C-terminal side by only two acidic residues, Asp4 and Glu5, located at positions $n + 2$ and $n + 3$, respectively. This situation fulfills the minimal consensus sequence but is not frequently found in CK2 substrates, which are characterized by an average of 5.2 acidic residues (*38*). Moreover, the presence of a stretch of eight consecutive lysines immediately C-terminal to Asp4 and Glu5 could be expected to hinder interaction of eIF2β with the catalytic subunit's network of basic residues, thereby interfering with correct substrate orientation (*11*).

To identify the features important for efficient phosphorylation of eIF2β, we synthesized a peptide corresponding to its first 22 residues (i.e., MSGDEMIFDPTMSKKKKKKKKP) and compared its time course of phosphorylation by the CK2 catalytic subunit and the holoenzyme, with the reference CK2 substrate peptide RRRADDSDDDDD used as a control (Figure 1). Results showed that under comparable conditions the eIF2β peptide was phosphorylated slightly better than the standard peptide by the holoenzyme. In contrast, it was very poorly phosphorylated by the isolated α subunit. Similar results were obtained by

Table 1: Kinetic Analysis of eIF2β-Derived Peptides as Substrates of CK2 Catalytic Subunit and Holoenzyme[a]

		CK2α			CK2$\alpha_2\beta_2$		
peptide	sequence	V_{max} (pmol/min)	K_m (mM)	efficiency (V_{max}/K_m)	V_{max} (pmol/min)	K_m (mM)	efficiency (V_{max}/K_m)
eIF2β[1−22]	M**S**GDEMIFDPTMSKKKKKKKKP	5.033	0.660	7.6	71.428	0.010	7142.8
eIF2β[1−22]A4A5	M**S**G**AA**MIFDPTMSKKKKKKKKP	nd	nd		nd	nd	
eIF2β[1−22]A9	M**S**GDEMIF**A**PTMSKKKKKKKKP	5.280	0.402	13.1	61.050	0.010	6105.0
eIF2β[1−11]A11	M**S**GDEMIFDPA	nd	nd		27.367	0.036	760.1
eIF2β[1−11]A11A9	M**S**GDEMIF**A**PA	nd	nd		23.651	0.041	576.8
eIF2β[1−11]A11A8	M**S**GDEMI**A**DPA	nd	nd		36.398	0.110	330.8
eIF2β[1−11]A11A8A9	M**S**GDEMI**AA**PA	nd	nd		30.103	0.176	171.0
standard	RRRADDSDDDDD	18.867	0.025	754.6	56.497	0.014	4035.5

[a] The data represent the mean of at least three independent experiments with SD never exceeding 12%. Kinetic parameters were determined as indicated in the Experimental Procedures section; nd, not determined due to undetectable phosphorylation. Residues whose displacement and/or substitution are discussed are shown in boldface.

replacing the α subunit with human recombinant α′ or with the catalytic α subunit from maize (data not shown). On the other hand, neither the α subunit nor the reconstituted holoenzyme showed significant activity toward an eIF2β peptide in which the acidic determinants at positions $n + 2$ and $n + 3$ were replaced by alanines, indicating that phosphorylation of eIF2β by CK2 indeed requires the presence of a canonical CK2 consensus sequence.

Table 1 reports the results of a kinetic analysis. The eIF2β peptide was phosphorylated more than 800-fold more efficiently by the holoenzyme than by the α subunit, with a 15-fold higher V_{max} and a 66-fold lower K_m (10 vs 660 μM). No detectable phosphorylation was observed with the alanine-substituted eIF2β peptide, even when added at a concentration >1 mM. It is noteworthy that, despite its reduced number of acidic determinants, the 22-residue eIF2β peptide displayed K_m and V_{max} values significantly better than those obtained for the standard peptide, whose phosphorylation is "optimized" by seven aspartyl residues surrounding the target serine. Taken together, these data demonstrate that the β subunit plays a crucial role in directing optimal phosphorylation of eIF2β.

Molecular Docking of the eIF2β[1−22] Peptide by CK2 Holoenzyme. In order to analyze the structural elements responsible for the binding of the eIF2β[1−22] peptide to CK2 holoenzyme, we first attempted to gain information on its secondary structure. To address this issue a circular dichroism (CD) analysis was performed in the far-UV region (see Figure 2A). Under conditions mimicking a physiological environment (i.e., PBS), the peptide yielded a spectrum dominated by a large negative minimum at about 200 nm, indicative of a predominantly random structure. The propensity of the peptide to adopt a more ordered structure was investigated by adding increasing amounts of trifluoroethanol (TFE), a solvent known to promote the formation of secondary structure in peptides and proteins. As shown in Figure 2A, TFE increased the helical character of the spectrum of the peptide. In particular, based on the magnitude of the ellipticity at 222 nm, the peptide exhibited a nearly 10% helical structure in the presence of 50% TFE. Since lysine is considered to be a helix maker according to secondary structure prediction programs (GOR4 (*39*), HNN (*40*), Jpred (*41*), AGADIR (*42*)), it can be reasonably inferred from the data that the eIF2β[1−22] peptide might assume a random coiled structure at its N-terminal tail which includes the phosphorylation site, followed by a short C-terminal α-helix motif, probably from K14 to K17.

On the basis of this information a computer-aided protein−protein docking analysis was performed by using the coordinates available for the human CK2 hetrotetramer (PDB code 1JWH) together with peptide secondary structure data generated from CD investigations and secondary structure predictions.

We first performed a set of docking runs with Zdock (*30*), Gramm (*31*), Bigger (*32*), and Escher (*33*) software, to find the best setup for each protocol, using the crystallographic data for the kinase−peptide complex as a training set. The structures obtained through the protein−protein docking approaches were highly superimposable with the crystallographic complexes (data not shown). We therefore used this validated strategy to explore all possible binding combinations between the eIF2β[1−22] peptide and the CK2 tetramer. As described in detail in Experimental Procedures, the first rigid docking phase and the second flexible minimization stage yielded the model illustrated in Figure 2B. In this model, the peptide is bound to the catalytic site of the α subunit and also extends with its C-terminal tail over the adjacent regulatory β subunit. Figure 2C shows details of the interactions that highlight two structural elements which reinforce the reliability of the resulting final model: first, the phosphorylatable Ser2 of the peptide faces the catalytic residue Asp156 of the CK2 α subunit and is therefore a position suitable for the phosphotransferase reaction; second, the acidic determinants located at positions $n + 2$ and $n + 3$ of the substrate are indeed in electrostatic contact with the basic residues Lys74 and Lys77 belonging to helix C of the kinase (at a distance of 2.4 and 2.5 Å, respectively), suggesting that the N-terminal moiety of the peptide is bound within the catalytic pocket in a canonical manner.

A few additional interesting interactions are disclosed by the model: (i) the basic stretch of eight lysines, located near Ser2 but protruding out of the catalytic site, makes contacts with the N-terminal acidic stretch of the regulatory β subunit, the same cluster which is proposed to be responsible for the downregulation of kinase activity (*43*) and for its responsiveness to polybasic compounds (*44*); (ii) the acidic residue Asp9, located at position $n + 7$ in the peptide, is positioned 2.5 Å from Lys33 and 2.8 Å from Arg47 of the regulatory β subunit; (iii) the aromatic ring of Phe8 of the peptide is in close proximity to that of Phe34 in the β subunit, forming a $\pi-\pi$ stacking interaction. Therefore, at least three molecular contacts appear to optimize binding of the substrate. These are mediated by the N-terminal region of the regulatory

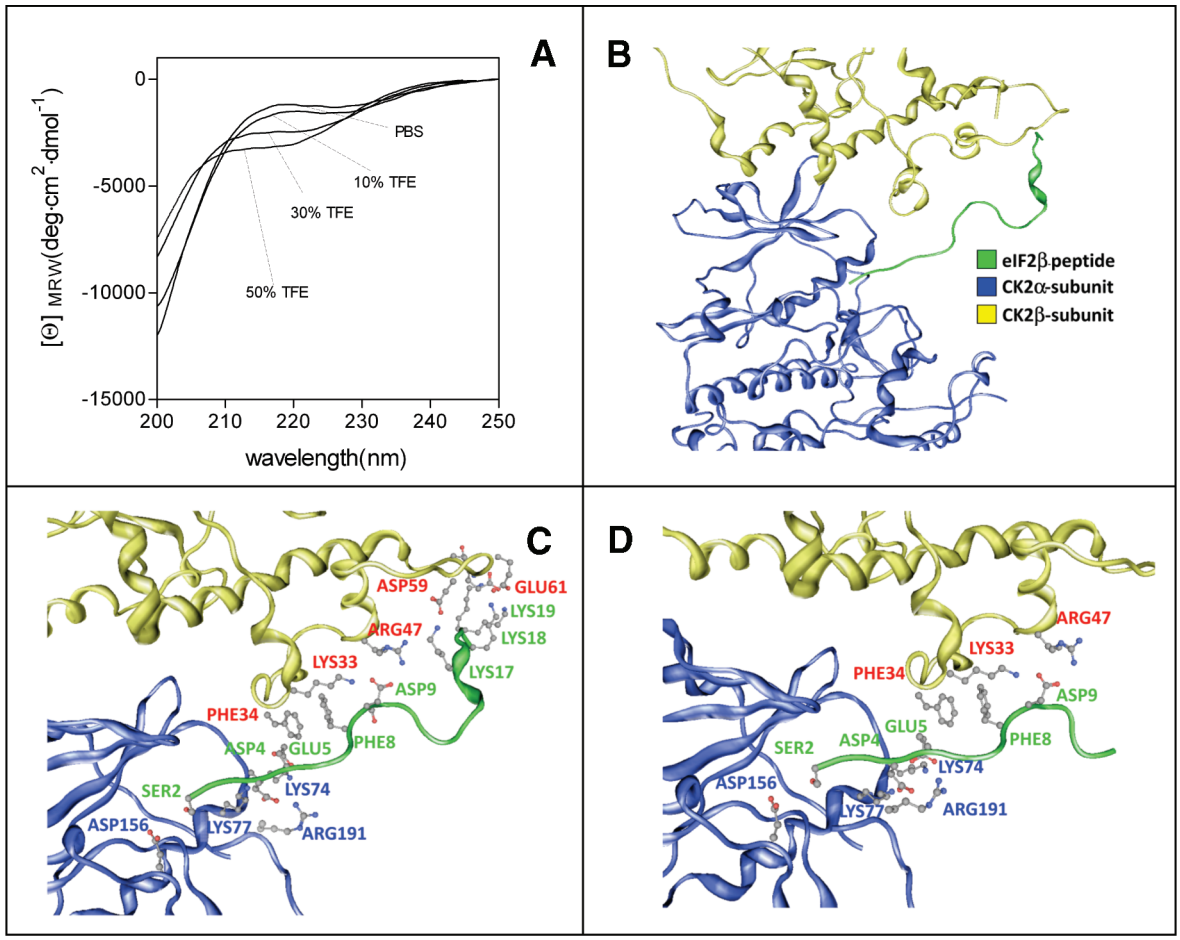

FIGURE 2: Structural analysis and protein−protein docking of the eIF2β peptide with CK2. Panel A shows the far-UV CD spectra of the peptide eIF2β[1−22]. All measurements were carried out at 25 °C in 10 mM sodium phosphate buffer (pH 7.4) as described in the Experimental Procedures. The effect of different concentrations of TFE on the conformation of the peptide was evaluated as indicated. Panel B shows the complex between CK2 holoenzyme 1JWH (the α subunit in blue, the β subunit in yellow) and the eIF2β[1−22] peptide (in green) obtained from the protein−protein docking approach. Panel C shows a magnification to highlight the molecular contacts between the peptide and the kinase. Labels refer to the residues of the peptide (in green) and the kinase (in blue and yellow for α and β subunits, respectively) implicated in crucial interactions. Panel D illustrates the complex obtained by docking the shorter derivative eIF2β[1−11] under identical conditions.

subunit which flanks the catalytic site and represents a continuation of the surface of the kinase that may interact with the specificity determinants located a short distance from the phosphorylation site.

The contribution of the β subunit to substrate recognition was also confirmed by performing the same docking analysis with a shorter eIF2β peptide devoid of the C-terminal basic stretch, i.e., peptide eIF2β[1−11]. As illustrated in Figure 2D, the binding model obtained for eIF2β[1−11] practically overlaps that adopted by the N-terminal moiety of the parent peptide but expectedly with weaker affinity given the absence of C-terminal interactions with the β subunit. It is important to note that by an identical computational docking analysis performed on the 1−22 or 1−11 eIF2β peptide and the free CK2 catalytic subunit the same complexes cannot be obtained (data not shown).

Validation of the Model. In order to assess the relevance of the structural elements highlighted by the theoretical model, we modified the parent eIF2β[1−22] peptide by either deleting its C-terminal lysyl stretch or by substituting Phe8 and Asp9 with alanines and compared the ability of these peptides to undergo phosphorylation by CK2 α and the holoenzyme. The results of these kinetic analyses are reported in Table 1. Results showed that none of the new peptides was phosphorylated by the free catalytic subunit. Furthermore, a remarkable decrease in phosphorylation by the holoenzyme was observed upon the deletion of the C-terminal basic stretch of lysines, with the decapeptide MSGDEMIFDPA displaying a 3.6-fold higher K_m and a 2.6-fold lower V_{max} and an overall 10-fold reduction in phosphorylation efficiency compared to the parent peptide. Replacement of eIF2β[1−11] residues Phe8 and Asp9 with alanines, either individually or in combination, resulted in a progressive increase in K_m. Its value rose to 176 μM with the double substitution, leading to a further reduction in the phosphorylation efficiency to a value of 171, i.e., about 42-fold lower than that initially observed with the parent peptide eIF2β[1−22]. Taken together, these experiments support the view that the molecular contacts between eIF2β[1−22] and the CK2 regulatory subunit indicated by the molecular docking approach play a fundamental role in determining the optimal phosphorylation of this peptide substrate. Particular importance is focused on the multiple electrostatic interactions of the lysyl stretch, whose deletion alone causes a 10-fold decrease in phosphorylation efficiency. In order to verify whether the same interactions observed with eIF2β 1−22 peptide were also occurring between CK2 β subunit and full size eIF2β, three mutants of the latter were generated.

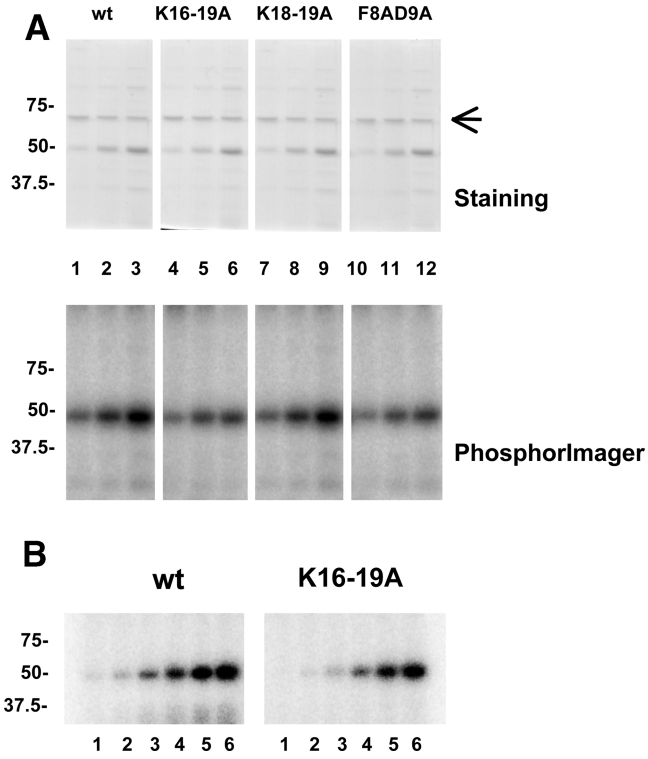

FIGURE 3: Dose-dependent phosphorylation of wild-type and mutated eIF2β by CK2 holoenzyme. Phosphorylation of the protein substrates (each at 0.75, 1.5, and 3 μM concentration) was performed and evaluated as described in the Experimental Procedures section by using recombinant CK2 reconstituted in vitro after mixing α and β subunits at 1:1 molar ratio. (A) Staining and PhosphorImager analysis of SDS−PAGE of wild-type eIF2β (lanes 1−3) and mutants K16−19A (lanes 4−6), K18−19A (lanes 7−9), and F8AD9A (lanes 10−12). The positions of external M_r markers are reported. The arrow indicates bovine serum albumin added, as a loading control, to the assays. (B) PhosphorImager analysis of phosphorylation kinetics performed at increasing concentrations (0.5, 1, 2, 3, 4, and 5 μM) of wild type and K16−19A mutant.

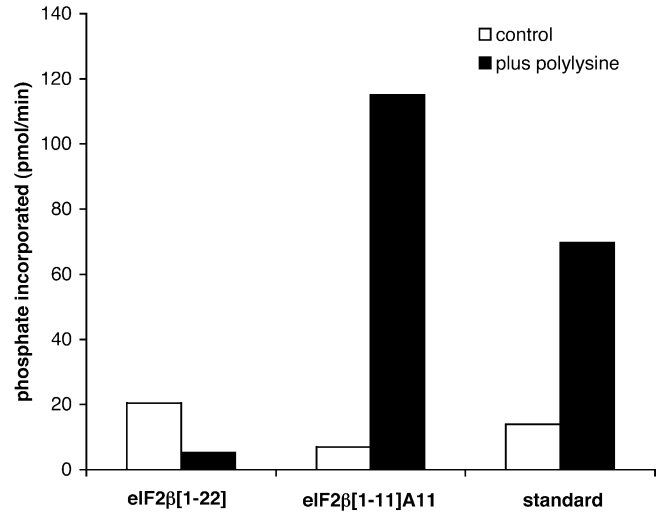

FIGURE 4: Opposite effects of polylysine on the phosphorylation of eIF2β peptides. The phosphorylation of eIF2β-derived peptides (200 μM) by native CK2 holoenzyme and evaluation of phosphate incorporation were performed as described in the Experimental Procedures. Polylysine was present at 330 nM where indicated. Phosphorylation data for the standard peptide RRRADDSDDDDD (100 μM) are also shown for comparison.

Two of them were aimed at altering the basic stretch downstream from Ser2 by replacing with alanines the 18−19 doublet and the whole 16−19 quartet of lysines, respectively. The third mutant was reproducing the effect of the double substitution with alanine of Phe8 and Asp9. The phosphorylation of these mutants by CK2 holoenzyme in comparison with wild-type eIF2β is illustrated in Figure 3A. Apparently none of the mutations cause dramatic alteration of the phosphorylation pattern. However, a reduced phosphorylation is observed with K16−19A and, to a lesser extent, with F8AD9A mutants while the double mutant K18−19A behaves similarly to wild-type eIF2β. It is important to note that none of the mutants were appreciably phosphorylated by CK2 α catalytic subunit (not shown). A kinetic analysis of the K16−19A mutant showed that, as in the case of eIF2β peptides, the decrease in phosphorylation efficiency with respect to wild-type eIF2β is mainly accounted for by increased K_m rather than by reduced V_{max} (Figure 3B).

Negative Influence of Polybasic Compounds on the Phosphorylation of eIF2β[1−22] Peptide by CK2 Holoenzyme. Assuming that, as suggested by modeling, the basic stretch in the eIF2β[1−22] peptide makes electrostatic contact with the negative cluster present in the N-terminal domain of the regulatory subunit, one would expect that stimulation by polybasic peptides targeting the same region of CK2 should be perturbed. This possibility was confirmed by results of an experiment carried out to test the effect of polylysine on phosphorylation of the eIF2β-derived peptides by CK2 holoenzyme (Figure 4). In fact, while phosphorylation of the standard peptide RRRADDSDDDDD was significantly stimulated by polylysine, phosphorylation of the eIF2β peptides was variably affected by polylysine depending on the presence of the lysyl stretch. In particular, while a more than 16-fold stimulation was observed with the short peptide 1−11 devoid of the lysyl stretch, phosphorylation of the parent peptide 1−22 was actually inhibited. As shown in Table 2, the opposite effect of polycations on the phosphorylation of eIF2β peptides is accounted for by changes in different kinetic constants. In the case of the short peptide eIF2β[1−11] polylysine affected mainly, if not exclusively, the V_{max}, which increased about 15-fold, the K_m remaining almost unchanged. On the contrary, in the presence of polylysine the larger peptide eIF2β[1−22] displayed a much higher K_m, which rose from 4.1 to 588 μM, reflecting a decrease in affinity comparable to that observed in the absence of the β subunit. Interpretation of these data leads us to conclude that engagement of the β subunit N-terminal acidic cluster by polylysine, while normally resulting in deinhibition of CK2 activity, prevents the binding of the eIF2β[1−22] peptide.

N-Terminal Altered β Subunit Fails To Stimulate Phosphorylation of eIF2β[1−22] Peptide. Evidence supporting the crucial role of the N-terminal region of the CK2 β subunit in ensuring optimal binding of the eIF2β peptide was also obtained through studies of substitution and deletion mutants of the CK2 regulatory β subunit. We first examined the effect of mutating residues shown by the computational analysis to actively interact with the eIF2β peptide, namely, the acidic D_{55}LE triplet and the basic residue R47. Both mutants showed no impairment in their ability to stimulate phosphorylation of a standard CK2 substrate compared to CK2α alone but failed to stimulate to a similar extent phosphorylation of the eIF2β peptide (Figure 5). Second, we took advantage of the fact that the dual effects of the regulatory

Table 2: Opposite Effects of Polylysine on the Phosphorylation Kinetics of eIF2β Peptides by CK2 Holoenzyme[a]

		control			+polylysine		
peptide	sequence	V_{max} (pmol)	K_m (mM)	efficiency (V_{max}/K_m)	V_{max} (pmol/min)	K_m (mM)	efficiency (V_{max}/K_m)
eIF2β[1−22]	MSGDEMIFDPTMSKKKKKKKKP	37.2	0.004	9300	9.0	0.588	15
eIF2β[1−11]A11	MSGDEMIFDPA	6.9	0.002	3450	99.7	0.004	24925

[a] The data represent the mean of at least three independent experiments with SD never exceeding 12%. Kinetic parameters were determined as indicated in the Experimental Procedures section.

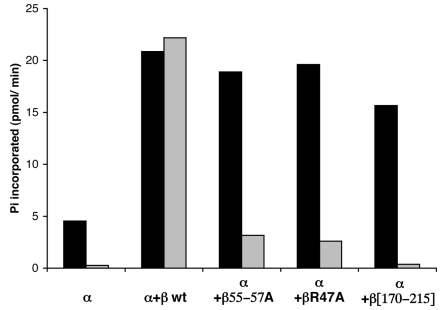

FIGURE 5: Phosphorylation of the eIF2β[1−22] peptide requires interaction with the N-terminal region of the β subunit. eIF2β[1−22] peptide (200 μM, gray bars) was phosphorylated as described in the Experimental Procedures using either free α subunit (0.5 pmol), α subunit plus wild-type β subunit (0.5 pmol), α subunit plus mutant βR47A (0.5 pmol), or α subunit plus an 800-fold molar excess of the truncated fragment β[170−215]. Phosphorylation data for the standard peptide (100 μM) are shown for comparison (black bars).

β subunit on the catalytic activity of the α subunit can be dissected by using large peptides reproducing its N- and C-terminal regions (*13*). In particular, the positive effect of the C-terminal domain is mimicked by a molar excess of a peptide spanning residues 175−215 which stimulated the basal activity (monitored with the standard peptide) to a degree comparable to that observed upon addition of full-length β subunit. This truncated form of the regulatory subunit, lacking the acidic N-terminal domain, is therefore still able to physically interact with the α subunit to form a rudimental complex (*45*) able to bind the substrate with an efficiency significantly higher than the α subunit alone. As shown in Figure 4, the truncated β subunit [175−215] entirely lost its stimulatory properties when the standard peptide substrate was replaced by the eIF2β[1−22] peptide. This outcome is fully consistent with our model showing that the eIF2β[1−22] peptide interacts with the N-terminal region of the β subunit, which is altered in the R47A and $DLE_{57}AAA$ mutants and is totally lacking in the truncated form β[175−215].

DISCUSSION

The regulatory function of the β subunit of protein kinase CK2 was first documented by the pioneering work of Cochet and Chambaz (*46*), who isolated the α and β subunits of bovine CK2 by preparative reversed polyacrylamide gel electrophoresis followed by renaturation of the proteins. Their investigations showed that the addition of the β subunit was required for maximal kinase activity from the α subunit. Later experiments performed by using recombinant proteins confirmed the β subunit's role in protecting the enzyme against proteolysis and denaturation promoted by heat and urea (*12*). Subsequent studies based on site-directed mutagenesis revealed a dual function of the β subunit, i.e., a positive effect mainly due to its C-terminal region and a negative effect attributable to its acidic N-terminal domain (*13, 14, 43, 44, 47*). This latter property suggested that the β subunit may function as an intrasteric pseudosubstrate capable of interacting with the positive charges of helix C located in the α subunit's catalytic site and competing with the acidic determinants near the substrate's phosphorylation site. The ability of polybasic compounds to stimulate the CK2 holoenzyme was ascribed to their interaction with the β subunit's acidic N-terminal domain, which would neutralize its pseudosubstrate activity.

Other observations, however, were not fully accounted for by this simplistic scenario. First, instead of exerting an inhibitory function, the acidic residues clustered in the β subunit's N-terminal domain were found to be absolutely required for efficient phosphorylation of the HIV-1 Rev protein (*22*) by interacting with a helix−loop−helix basic motif. Second, the 3D structure of the human CK2 heterotetramer showed that the acidic loop of the β subunit is >30 Å distant from the nearest basic cluster of the catalytic subunit, thus ruling out the possibility of a truly intramolecular downregulation (*15*). This latter finding disclosed a crucial role for the supramolecular organization of CK2 multimers in which the interaction between the acidic loop of β subunits and the basic cluster of α subunits might occur between subunits of different complexes in the autophosphorylation process taking place at β subunit Ser2 (*8*). More recently, the phosphorylation features of another CK2 substrate, the eukaryotic translation-initiation factor eIF2β, were found to be reminiscent of those displayed by HIV-1 Rev (*25*) for being entirely dependent on the heterotetrameric structure of CK2. We therefore carried out a more detailed investigation of the role played by CK2β subunit during eIF2β phosphorylation.

The work presented in this paper demonstrates that the N-terminal segment of eIF2β, which bears both the phosphorylation site and a downstream stretch of eight consecutive lysines, behaves like the full-length protein in contrast with previous findings observed in the case of HIV-1 Rev protein (*22*). In fact, a synthetic peptide reproducing this segment (residues 1−22) is phosphorylated only by the oligomeric CK2 holoenzyme, with quite favorable kinetic constants that were wholly unexpected considering the reduced number of acidic determinants located downstream from the target Ser2. In contrast, the free catalytic subunit phosphorylated the same peptide with negligible efficiency. In particular, the affinity of CK2 for the peptide appeared to greatly benefit from the presence of the β subunit, its K_m value being more than 2 orders of magnitude lower than that observed with the isolated catalytic subunit. This prompted us to analyze the mode of binding of the eIF2β peptide to the kinase through a computational approach based on

protein−protein docking techniques. The complex with the highest score confirmed the canonical interaction between the substrate's acidic determinant located at the crucial position $n + 3$ with respect to Ser2 and the basic cluster of helix C of the α subunit. In addition, this analysis highlighted three new and unexpected molecular contacts with the regulatory β subunit. The first, electrostatic in nature, connects the C-terminal basic cluster of the peptide to the 55−64 acidic domain of the β subunit. A second contact involves two apolar residues, i.e., Phe8 of the peptide and Phe34 of the β subunit, and the third interaction consists of an electrostatic attraction between Asp9 of the peptide and the two basic residues Lys33 and Arg47 of the β subunit. The relative importance of each of these interactions, which synergistically contribute to the optimal binding of the peptide substrate, was investigated by examining the phosphorylation of a number of shortened and variably substituted eIF2β derivatives as well as three mutants of full-length eIF2β. Although of minor importance in the case of the parent eIF2β[1−22] peptide, both the aromatic ring of Phe at position $n + 6$ and the negative charge of Asp at position $n + 7$ apparently significantly increase the affinity of the shortened peptide for the heterotetrameric kinase, in which the interaction with the acidic domain of the β subunit is absent. On the other hand, the critical role of the N-terminal domain of the β subunit is outlined by the effect of polybasic compounds and by the activities observed using mutated versions of the β subunit altered either in the acidic domain or residue R47 or entirely devoid of its N-terminal region. In fact, all of these modifications resulted in dramatically impaired phosphorylation efficiency. The N-terminal acidic domain of the regulatory subunit therefore appears to play a critical role in this interaction. To the same conclusions lead also the experiments performed with full-length eIF2β mutated in the N-terminal basic stretch which demonstrate a significantly reduced affinity when four out of eight lysines (mutant K16−19A) were replaced by alanines. It should be underlined, however, that the minimum consensus sequence specified by at least one acidic residue at position $n + 3$ is still a fundamental requirement, since phosphorylation of the eIF2β[1−22] peptide, whose binding is largely supported by the regulatory subunit, is completely abolished upon replacement of the two acidic determinants at position $n + 2$ and $n + 3$ with alanines.

Therefore, it is possible to conclude that, in the case of eIF2β, the recognition of specificity determinants located downstream from the CK2 phosphorylation sites is mediated by both the catalytic and regulatory subunits and exploits different types of interactions in a synergistic manner. Such a mode of substrate recognition may not be restricted to eIF2β, since the phosphorylation sites identified in many CK2 substrates display extended stretches of C-terminal acidic residues able to interact with the β subunit (*38*). In fact, about 70% of the CK2 phosphorylation sites examined possess at least one acidic residue at positions spanning from $n + 6$ to $n + 8$ which, according to the data presented in this work, are potentially able to interact with the β subunit and to strengthen the binding of the substrate. It will therefore be interesting to determine whether, as shown for eIF2β, other CK2 substrates also exploit specificity determinants located beyond the canonical positions strictly controlled by the catalytic subunit. In fact, examination of the primary structure around the atypical phosphorylation sites identified in some substrates apparently not fulfilling the consensus sequence for CK2 (e.g., caldesmon, IGFBP1, NDPKA, and nonmuscle myosin B) reveals the presence of multiple acidic determinants at positions spanning from $n + 5$ to $n + 7$ which could be sufficient to circumvent, at least in these cases, the lack of a minimum consensus for CK2. Similarly, basic residues downstream from the canonical consensus of CK2 phosphorylation sites could represent additional elements optimizing the binding of substrates with a reduced number of acidic determinants. To gain a clear picture of the contribution of this and of other potential molecular contacts outside of the catalytic pocket, it will be necessary to solve the structure of CK2 complexed with substrate molecules or substrate competitive inhibitors as well as improve our understanding of the mechanism(s) underlying the relationship between kinase multimerization and substrate phosphorylation.

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