

# Unique features of HIV-1 Rev protein phosphorylation by protein kinase CK2 ('casein kinase-2')

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Received 28 July 2000; accepted 6 August 2000

Edited by Shmuel Shaltiel

**Abstract** The HIV-1 Rev transactivator is phosphorylated *in vitro* by protein kinase CK2 at two residues, Ser-5 and Ser-8; these sites are also phosphorylated *in vivo*. Here we show that the mechanism by which CK2 phosphorylates Rev is unique in several respects, notably: (i) it is fully dependent on the regulatory,  $\beta$ -subunit of CK2; (ii) it relies on the integrity of an acidic stretch of CK2 $\beta$  which down-regulates the phosphorylation of other substrates; (iii) it is inhibited in a dose-dependent manner by polyamines and other polycationic effectors that normally stimulate CK2 activity. In contrast, a peptide corresponding to the amino-terminal 26 amino acids of Rev, including the phosphoacceptor site, is readily phosphorylated by the catalytic subunit of CK2 even in the absence of the  $\beta$ -subunit. These data, in conjunction with the observation that two functionally inactive derivatives of Rev with mutations in its helix-loop-helix motif are refractory to phosphorylation, indicate the phosphorylation of Rev by CK2 relies on conformational features of distinct regions that are also required for the transactivator's biological activity. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Casein kinase-2; Protein kinase; Protein phosphorylation; Human immunodeficiency virus type-1; Rev

## 1. Introduction

The acronym CK2, derived from the misnomer 'casein kinase-2', refers to a Ser/Thr protein kinase physiologically unrelated to casein, whose targeting is specified by multiple acidic residues and is able to use either GTP or ATP as a phosphate donor (reviewed in [1–3]). In addition to phosphorylating Ser/Thr residues on a variety of substrates, it was recently shown that CK2 is also able to phosphorylate tyrosyl residues [4,5]. However, the most intriguing feature of CK2 is probably its enigmatic mode of regulation. At variance with the majority of protein kinases, the catalytic  $\alpha$  and  $\alpha'$  subunits of CK2 are constitutively active in the absence of second messengers, activating subunits or phosphorylation events.

Moreover, association of the  $\alpha$  and  $\alpha'$  subunits with two regulatory  $\beta$ -subunits, yielding a stable heterotetrameric holoenzyme, has different effects on catalytic activity depending on the substrate; many substrates, notably small peptides, are more readily phosphorylated in the presence of  $\beta$  subunits, while the phosphorylation of other substrates (e.g. calmodulin) is inhibited. This dual role of the  $\beta$ -subunit is believed to reflect the presence of distinct domains playing different and in some respects opposite roles. These domains have been mapped by generating  $\beta$  mutants defective in either up- or down-regulatory properties [6–8] and synthetic fragments of the  $\beta$  subunit retaining only some of the properties of the full-size protein [9,10]. Results of these studies demonstrated that down-regulation is mediated by acidic residues concentrated in the N-terminal part of the molecule, whose mutation to alanines suppresses the ability of the  $\beta$  subunit to inhibit calmodulin phosphorylation, while it stimulates catalytic activity toward peptide substrates [6,8]. This acidic region is also responsible for the interaction of the  $\beta$  subunit with polycationic molecules, such as histones, polylysine [8] and polyamines [11], which are known to stimulate the activity of the CK2 holoenzyme at least in part by neutralizing the down-regulatory potential of the  $\beta$  subunit.

Despite these fine-tuned modes of regulation, it must be assumed that CK2 is always constitutively active within the living cell: this apparent paradox may explain the extraordinary pleiotropy of CK2, whose growing list of substrates currently includes more than 200 proteins [12]. It is conceivable therefore that a discrete level of CK2 activity is required at all stages of differentiation and under all metabolic conditions. It is also possible that its constitutive activity underlies the imputed role of CK2 in pathogenic events, notably neoplasias and infectious diseases (reviewed in [3,13]). As far as the latter are concerned, it is remarkable that many viruses exploit host cell CK2 to phosphorylate their own proteins. This makes CK2 a potential target for antiviral as well as antineoplastic therapies [14].

The list of CK2 substrates identified thus far includes human immunodeficiency virus type-1 (HIV-1) proteins, i.e. Vpu and Rev, whose CK2-mediated phosphorylation was established both *in vitro* and *in vivo* [15,16]. While the phosphorylation of Vpu has been correlated with its ability to interact with CD4 [17,18], a crucial step in the viral infection strategy, nothing is known about the significance of Rev phosphorylation, which takes place at two residues close to the N-terminus of the protein, Ser-5 and Ser-8, the latter of which fulfils the consensus sequence for CK2 targeting and, once phosphory-

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**Abbreviations:** CK2, casein kinase-2; HIV-1, human immunodeficiency virus type-1

lated, creates the consensus for the phosphorylation of Ser-5 [16]. These phosphorylation sites lie adjacent to an arginine-rich sequence spanning amino acids 35–50 that is required for the ability of Rev to accumulate in the nucleus and to bind to RNA, two properties that are crucial to its functional activity as a post-transcriptional regulatory factor (reviewed in [19]). Intriguingly, this domain is also responsible for the ability of Rev to stimulate CK2 activity toward other protein substrates [20]. In an attempt to shed light on the mechanism by which CK2 phosphorylates Rev, we have investigated the effect of modulators of CK2 activity and of Rev mutations which are known to impair its function. The results, presented in this report, highlight the unique behavior of Rev among the known substrates of CK2 and raise the possibility of selectively inhibiting Rev phosphorylation under conditions that do not reduce the activity CK2 toward its cellular targets.

## 2. Materials and methods

### 2.1. Materials

Recombinant  $\alpha$  and  $\beta$  subunits of either wild type or mutated human CK2 were obtained as previously detailed [21,22]. Native CK2 purified from rat liver was prepared as described [23]. Wild type and mutant Rev proteins were produced in bacteria as GST fusion proteins from pGEX2T-derived plasmids, isolated by affinity chromatography using glutathione-Sepharose, and then eluted using glutathione according to standard protocols [24]. The strategy used to clone plasmids encoding GST-Rev and GST-Rev38,39R-L is described elsewhere [25]. To clone the plasmid expressing GST-Rev $\Delta$ loop, a pair of PCR fragments encoding sequences amino-terminal and carboxy-terminal to the loop were ligated and then subcloned into the pGEX2T backbone; this cloning approach deleted residues 24–34 and also inserted a leucine codon after residue 35.

A synthetic peptide corresponding to the 26 amino-terminal residues of Rev was prepared on a 431A (Applied Biosystems) peptide synthesizer using (fluorenylmethoxy)carbonyl (Fmoc) chemistry with an *N*-methylpyrrolidone solvent system [26]. Chain assembly was performed on 0.1 mmol of Rink amide AM resin (0.69 mmol/g) (Novabiochem) using Fmoc-protected amino acids activated with a mixture of 2-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, *N*-hydroxybenzotriazole and *N*-ethyl-diisopropylamine in dimethylformamide. Side chain protecting groups were removed during cleavage with a solution containing 95% (v/v) trifluoroacetic acid, 2.5% (v/v) triisopropylsilane and 2.5% (v/v) water. After cleavage, peptides were purified to homogeneity by reversed-phase high-performance liquid chromatography with a linear gradient of 0–60% acetonitrile. Purity of the synthetic peptides was assessed by matrix-assisted laser desorption ionization mass spectrometry.

Synthetic peptides reproducing residues 67–93 of Inhibitor-2 and the central helix residues 68–92 of calmodulin were prepared as previously described [27,28].

### 2.2. Phosphorylation assay

Phosphorylation experiments were performed at 37°C for 10 min in a 25  $\mu$ l final volume containing 50 mM Tris-HCl pH 7.5, 12 mM MgCl<sub>2</sub>, 100 mM NaCl, 20  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (specific activity 500–1000 cpm/pmol) and GST-Rev fusion proteins or peptides. The concentra-

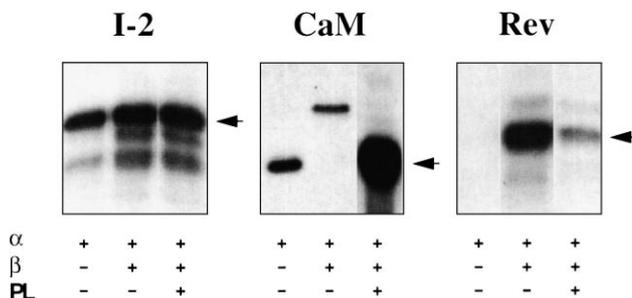


Fig. 1. Distinct effects of the  $\beta$  subunit on the phosphorylation of CK2 protein substrates. Recombinant Inhibitor-2 (I-2), calmodulin (CaM) and Rev (2  $\mu$ g) were incubated with [<sup>32</sup>P]ATP in the presence of recombinant CK2  $\alpha$  subunit (5 pmol) either alone or previously mixed with an equimolar amount of  $\beta$  subunit and then subjected to SDS-PAGE through a 15% gel (11% in the case of CaM and Rev), all as described in Section 2. Polylysine, when present, was 336 nM. Shown is the resulting autoradiogram with arrows indicating the positions of I-2, CaM and Rev, with  $M_r$  31 kDa, 15.6 kDa and 19 kDa, respectively. The upper band visible in the central lane of the CaM panel is due to autophosphorylation of the  $\beta$  subunit ( $M_r$  25 kDa) which is inhibited by polylysine.

tions of the phosphorylatable substrate and the enzyme are specified in the figure legends and tables. The reaction was initiated with the addition of the kinase and was stopped by cooling in ice. Protein samples were then subjected to SDS-PAGE according to Laemmli [29], followed by staining with Coomassie brilliant blue and either autoradiography or radioanalytic scanning on a Canberra-Packard Instant Imager. Phosphorylation of synthetic peptides was measured using the phosphocellulose paper procedure [30].

## 3. Results

The substrate-dependent effect of the  $\beta$  subunit on CK2 activity is exemplified in Fig. 1, which compares the phosphorylation of three physiological substrates catalyzed by either the catalytic  $\alpha$  subunit or the holoenzyme reconstituted by combining equimolar amounts of the  $\alpha$  and the  $\beta$  subunits. Inhibitor-2 of protein phosphatase 1 (I-2) is readily phosphorylated by CK2 $\alpha$  alone and even more rapidly by the holoenzyme, denoting a moderate stimulatory effect of the  $\beta$  subunit, as normally observed with specific peptide substrates of CK2. In sharp contrast, calmodulin is phosphorylated by CK2 $\alpha$  but not to any appreciable extent by the CK2 holoenzyme, highlighting the predominance of the negative regulatory effects of the  $\beta$  subunit, as has also been reported for a few other protein substrates of CK2 (see [12]). The behavior of Rev differs from that of I-2 and is opposite to that of calmodulin: phosphorylation, almost undetectable in the presence of CK2 $\alpha$  alone, is triggered by the  $\beta$  subunit upon holoenzyme reconstitution. This unique behavior of Rev, as well as the opposite

Table 1

CK2-catalyzed phosphorylation of synthetic peptides spanning the phosphoacceptor sites of Rev, calmodulin and Inhibitor-2 is stimulated to a similar extent by the  $\beta$  subunit

Peptide	Phosphorylation rate (cpm) by		Stimulation (fold)
	$\alpha$	$\alpha_2\beta_2$	
Rev(1–26) <u>MAGR</u> <u>SGDS</u> DEELIRTVRLIKLLYQSN	3 106	12 240	3.94
CaM(68–92) <u>FLTMMARKMKD</u> <u>TD</u> SEEEIREFRVF	6 016	35 075	5.83
I-2(67–93) <u>I</u> DEPSTPYHSMIGDDDDAY <u>SD</u> TETTEA	17 612	42 798	2.43
Standard RRRRAADSDDDDD	11 572	48 132	4.15

Peptides (40  $\mu$ M) were phosphorylated and <sup>32</sup>P incorporation was quantified as described in Section 2. The residues undergoing CK2-catalyzed phosphorylation are underlined. Values are means of at least three determinations with a standard error less than 15%.

property exhibited by calmodulin, disappears when the full-size proteins are replaced by synthetic peptides spanning their phosphoacceptor sites. In fact, as shown in Table 1, phosphorylation of peptides derived from Rev, calmodulin and I-2 as well as the standard peptide substrate  $R_3A_2DSD_5$  (routinely used for monitoring CK2 activity) is stimulated to a similar extent in the presence of the  $\beta$  subunit.

Besides its absolute requirement for the  $\beta$  subunit, another peculiarity of Rev phosphorylation is its inhibition by polycationic compounds which are generally considered stimulators of CK2 activity. As shown in Fig. 1, while polylysine slightly increases the phosphorylation of I-2 and dramatically enhances that of calmodulin by the CK2 holoenzyme, it exerts a detrimental effect on Rev phosphorylation. Phosphorylation of Rev is also inhibited in a dose-dependent manner by spermine, another polycation which is believed to be a physiological activator of CK2 [31] (Fig. 2A) and which under comparable conditions actually stimulates the phosphorylation of  $\beta$ -casein. The effects of spermine [11] and polylysine [8] are both mediated by the  $\beta$  subunit of CK2. In contrast the CK2 inhibitor heparin directly affects the catalytic subunit [21]. It may be worth noting in this connection that the response of Rev phosphorylation to heparin is regular (Fig. 2B).

It has been shown that stimulation of CK2 by polycations reflects, at least partially, their ability to interact with and neutralize an acidic region in the N-terminal domain of the  $\beta$  subunit, which exerts a down-regulating effect by interacting with the peptide substrate binding site of the  $\alpha$  subunit [32]. Consequently a mutant of the  $\beta$  subunit in which two crucial acidic residues of this region were replaced by neutral amino acids displays both hyperstimulation of catalytic activity [6]

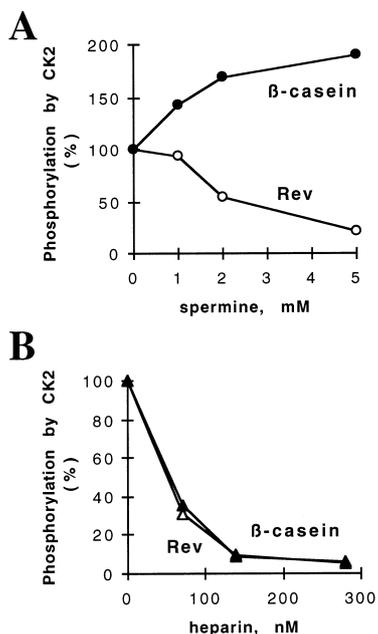


Fig. 2. Inhibition of Rev phosphorylation (open symbols) by spermine (A) and heparin (B). For the sake of comparison the phosphorylation of  $\beta$ -casein (filled symbols), which is enhanced by spermine, is also shown. Conditions are those detailed in the legend of Fig. 1 and in Section 2 except for the concentration of  $MgCl_2$  which was 2 mM. Phosphorylation is expressed as percent of controls in the absence of effectors, corresponding to 4.6 and 18.4 pmol for Rev and  $\beta$ -casein, respectively. Identical results were obtained with native CK2 purified from rat liver instead of the recombinant holoenzyme reconstituted in vitro (not shown).

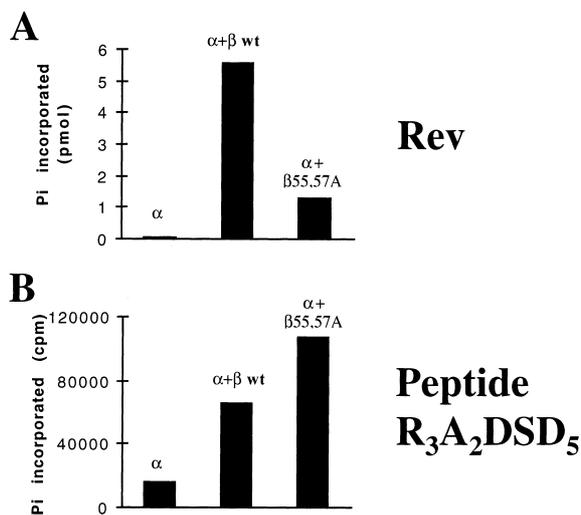


Fig. 3. Opposite effects of mutant  $\beta 55,57A$  on CK2-catalyzed phosphorylation of Rev (A) and of the synthetic peptide  $R_3A_2DSD_5$  (B). Rev protein (2  $\mu$ g) and the synthetic peptide RRRRAADSDDDDD (100  $\mu$ M) were phosphorylated under conditions described in the legend to Fig. 1 by recombinant CK2  $\alpha$  subunit either alone or previously mixed with an equimolar amount of wild type or mutated  $\beta$  subunit. The phosphorylation of Rev was determined after SDS-PAGE while that of the synthetic peptide was evaluated by separation on phosphocellulose paper [30].

and reduced susceptibility to further stimulation by polylysine [8]. As expected, this  $\beta$  subunit mutant hyperstimulates phosphorylation of the synthetic peptide  $R_3A_2DSD_5$  (Fig. 3B). In contrast, the same mutant displays a reduced ability to stimulate phosphorylation of Rev compared to the wild type subunit (Fig. 3A).

In summary, the phosphorylation of Rev by CK2 is unique in at least three respects: (i) it is fully dependent on the presence of the  $\beta$  subunit; (ii) it is up-regulated by acidic residues of the  $\beta$  subunit that normally down-regulate CK2 activity;

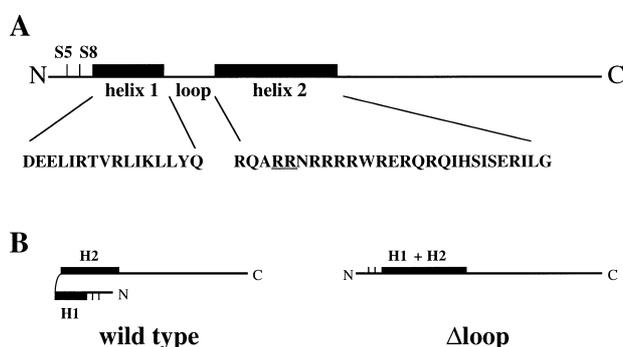


Fig. 4. Proposed conformational models of wild type Rev and Rev $\Delta$ loop. In A the schematic representation of wild type Rev is shown with the highlighted structural helix 1-loop-helix 2 motif. Basic residues R38 and R39, replaced by leucine in the mutant Rev38,39R-L, are underlined. Phosphoacceptor sites Ser-5 and Ser-8 are also indicated. In B the flexible model of the wild type Rev helix-loop-helix motif allowing the simultaneous binding to the catalytic site of CK2 $\alpha$  and to the acidic domain of CK2 $\beta$  subunit is proposed in comparison with the rigid, uninterrupted helix predicted for mutant Rev $\Delta$ loop. The secondary structures of wild type Rev and Rev $\Delta$ loop were predicted using the PHD sec method [37,38], available over the Internet (<http://dodo.cpmc.columbia.edu/predict-protein/>).

(iii) it is inhibited, rather than stimulated by polycationic effectors.

The overall conformation of the Rev protein apparently plays a role in conferring atypical responsiveness to  $\beta$  regulation, as demonstrated by the observation that a synthetic fragment of Rev including the phosphoacceptor sites (residues 1–26) is no longer dependent on the  $\beta$  subunit for efficient phosphorylation (see Table 1). Two structural features of Rev that could account for its atypical mode of phosphorylation by CK2 are a basic stretch (residues 35–50) that has been implicated in the observed stimulation of CK2 catalytic activity by Rev [20] and could interact with the acidic cluster in the N-terminal domain of the  $\beta$  subunit, and a helix 1-loop-helix 2 motif that is likely to play a key role in conferring a biologically active conformation [33,34]. Recent studies demonstrated that the loop is essential for Rev function (D'Agostino et al., unpublished results) and led to the proposal that it acts as a flexible hinge that allows correct alignment of the two helices, which might in turn place the polycation-like basic stretch in close proximity to Ser-5 and Ser-8 (see Fig. 4).

To assess the influence these two structural motifs might have on the phosphorylation of Rev by CK2, two mutants in this regions were compared to wild type Rev for their ability to undergo phosphorylation by the  $\alpha$  subunit and holoenzyme. The first mutant, named Rev38,39R-L, contains leucines in place of the first pair of arginines of the basic cluster. The second mutant, named Rev $\Delta$ loop, has a deletion encompassing the entire loop linking helices 1 and 2. This mutation is predicted to give rise to a rigid uninterrupted helix generated by the fusion of helices 1 and 2 (D'Agostino et al., unpublished results; see also Fig. 4). As shown in Table 2, both mutants are inefficiently phosphorylated by CK2 holoenzyme, although in the presence of the  $\alpha$  subunit alone they undergo a weak phosphorylation which is not detectable with Rev wild type. These data corroborate the idea that the tertiary structure of the protein plays a key role in the mechanism by which Rev is recognized and/or phosphorylated by CK2.

#### 4. Discussion

Our findings outline a number of features that distinguish Rev from other CK2 substrates studied thus far. First, Rev is not phosphorylated to any appreciable extent by the isolated catalytic subunit of CK2, in contrast to all other substrates tested so far, toward which CK2 $\alpha$  is constitutively active. Consequently, phosphorylation of Rev by CK2 is entirely dependent on the regulatory  $\beta$  subunits, which combine with the

catalytic subunits, yielding a very stable holoenzyme with the heterotetrameric  $\alpha_2\beta_2$  structure [35]. Although strong up-regulation in the presence of the  $\beta$  subunit has been reported for a few other substrates, e.g. CREB and MS2-E7 [36], the nearly absolute requirement for the  $\beta$  subunit is unique to Rev. Third, the acidic residues clustered in the N-terminal domain of the  $\beta$  subunit, which are normally responsible for down-regulation, are instead required for efficient Rev phosphorylation. Fourth, phosphorylation of Rev by the CK2 holoenzyme is inhibited by polybasic peptides and by polyamines which conversely behave as activators in the context of all the other CK2 substrates tested thus far. Such a behavior, especially as far as inhibition by spermine is concerned, subverts our current opinion about polyamines, which are considered physiological activators of CK2 [31]. This leads to the prediction that under physiological conditions where the concentration of spermine increases and consequently the activity of CK2 toward most of its targets is enhanced, the phosphorylation of HIV-1 Rev protein is conversely repressed. Assuming that Rev phosphorylation is essential to viral replication (yet to be proven), the above observation could also have practical applications, as it suggests the possibility of arresting or inhibiting virus infection by increasing the intracellular spermine concentration. It is also predictable that conditions promoting deep oscillations in spermine concentration might impinge on the level of Rev phosphorylation and possibly influence susceptibility to HIV-1 infection.

The unique behavior of Rev with respect to CK2-mediated phosphorylation is dependent, at least in part, on its tertiary structure. This conclusion is supported by two lines of evidence: first, a 26-mer peptide encompassing the Rev phosphoacceptor site (Ser-5 and Ser-8) has lost the most peculiar feature of Rev phosphorylation, namely the absolute requirement for the  $\beta$  subunit; secondly, phosphorylation of Rev by the CK2 holoenzyme is dramatically reduced by two structural modifications (i.e. deletion of the loop and the 38,39R-L mutation) which do not directly affect the phosphoacceptor site. It has to be assumed therefore that although the phosphoacceptor site fulfils the consensus for CK2 (S-x-x-D/E/Sp) and consequently is readily phosphorylated by CK2 $\alpha$  in the context of the 1–26 peptide, the overall conformation of the protein renders the site inaccessible or unsuitable in the absence of the  $\beta$  subunits.

Although the mechanism by which the  $\beta$  subunit assists Rev phosphorylation is currently a matter of conjecture, a direct interaction between the  $\beta$  subunit and Rev is strongly supported by the observation that a  $\beta$  subunit mutation which up-regulates the basal catalytic activity of CK2 holoenzyme (i.e. the ED55,57  $\rightarrow$  AA substitution) is instead detrimental to Rev phosphorylation. This suggests that a productive interaction of Rev with the CK2 holoenzyme requires those acidic residues of the  $\beta$  subunit which are otherwise committed to down-regulation of the catalytic site. It is quite possible that Rev's basic cluster of residues at the beginning of helix 2 represents the counterpart to the acidic residues of the  $\beta$  subunit, as the substitution of R38 and R39 by Leu greatly reduces its phosphorylation. This interaction may also represent the mechanism by which Rev stimulates the basal activity of the CK2 holoenzyme [20]: upon engagement with Rev, the acidic cluster of the  $\beta$  subunit would no longer be available for down-regulation. Likewise, inhibition of Rev phosphorylation by polycationic compounds can be explained by a com-

Table 2  
Phosphorylation of wild type and mutated Rev protein by the  $\alpha$  catalytic subunit and by in vitro reconstituted CK2 holoenzyme

Substrate	Phosphorylation rate (pmol) by	
	$\alpha$	$\alpha_2\beta_2$
GST-Rev wild type	N.D.	6.57
GST-Rev38,39R-L	0.34	0.50
GST-Rev $\Delta$ loop	0.18	0.24

Wild type and mutated proteins (2  $\mu$ g) were phosphorylated and  $^{32}$ P incorporation was quantified as described in the legend of Fig. 1. N.D., not detectable as radiolabeled SDS-PAGE band. The data refer to the mean of values obtained from three independent experiments with S.D. not exceeding 13%.

petitive mechanism that counteracts the interaction of Rev with CK2 $\beta$ . Therefore, paradoxically, although normally specified by a negatively charged consensus sequence on the substrate, recognition of Rev by CK2 would also critically require basic residues that are relatively far away from the phosphoacceptor site along the primary structure. It is predictable however that in the folded structure of Rev these basic residues are brought closer to the phosphorylatable residues by the juxtaposition of helices 1 and 2, made possible by the flexibility of the spacer loop (see Fig. 4). The detrimental effect of the deletion of this loop on Rev phosphorylation (see Table 2) is quite consistent with this scenario: as outlined in Fig. 4, this deletion is expected to give rise to an uninterrupted  $\alpha$  helix generated by the fusion of helices 1 and 2, with a consequent gross alteration in the spatial relationship between the phosphoacceptor site and the critical basic cluster. Intriguingly, this same deletion also destroys Rev function in vivo (D'Agostino et al., unpublished results). Based on this observation, in conjunction with the in vivo occurrence of Rev phosphorylation at the same residues affected in vitro by CK2 [16] it would be tempting to hypothesize a correlation between CK2-mediated phosphorylation and Rev functional activity. However, no direct evidence for such a functional correlation is available to date. On the other hand, the possibility should also be taken into account that phosphorylation by CK2 affects other properties of Rev, such as stability or propensity to multimerize, by analogy with the observed consequences of Rev phosphorylation at another site [39]. In conclusion, while a cause-effect relationship between Rev phosphorylation and its biological activity remains to be established, our data show that CK2-mediated phosphorylation and functional activity of Rev have similar tertiary structure requirements and are controlled by overlapping domains.

**Acknowledgements:** We thank T. Ferro for expert technical assistance. Financial support was provided by the Italian Ministero della Sanità (Progetto AIDS, Istituto Superiore di Sanità), the European Union (BioMed.2 BMH4-CT96-0047), the Armenise-Harvard Foundation, M.U.R.S.T. (PRIN97) and the CNR (97.03614.PS14 and T.P. on Biotechnology).

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