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Phosphorylation of HIV-1 Rev Protein: Implication of Protein Kinase CK2 and Pro-Directed Kinases

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HIV-1 Rev transactivator is readily phosphorylated at separate regions by protein kinase CK2 and MAP kinase. Protein kinase CK1 cannot replace CK2 as phosphorylating agent and cdc2 only slowly phosphorylates Rev at one of the two sites affected by MAP kinase. Mutational analysis shows that Ser-8 and, to a lesser extent, Ser-5 are phosphorylated by CK2. In contrast, a mutation (R_{14}TV → EED) which suppresses Rev activity dramatically enhances Rev phosphorylation either *in vitro* by CK2 or *in vivo*, suggesting that phosphorylation by CK2 could play a role in Rev down-regulation.

The Rev gene product of human immunodeficiency virus type 1 (HIV-1) is a 116 amino acid, predominantly nucleolar [1] protein that is essential for HIV-1 replication [2, 3]. Rev is a post-transcriptional activator of the unspliced and singly spliced viral mRNAs expression that exerts its effects via binding [4] to an RNA stem-loop structure termed the Rev-responsive element (RRE), located in the env gene [5]. Rev rescues these mRNAs from splicing and promotes their stability and transport to the cytoplasm, where they are translated into the late viral proteins (for reviews of the current knowledge of Rev function, see refs. [6, 7]).

Mutational analyses have defined regions that are essential for biological properties of Rev, including RRE binding, nucleolar localization, multimerization and transactivating competence. These studies have highlighted crucial contributions of the N terminal region, with special reference to an arginine-rich sequence spanning amino acids 35-50 [8-10] and a domain spanning amino acids 75-84 that is essential for the proteins biological activity [11].

Rev is known to be phosphorylated on serine residues [12-14]. Although mutational studies are consistent with the idea that phosphorylation of Rev is not required for activity [11, 12] it is quite conceivable that it reflects a regulatory mechanism of its biological properties. While the host cell protein kinase(s) responsible for Rev phosphorylation are still unknown, the sequences surrounding serine residues that appear to undergo phosphorylation suggest potential candidates (see Figure 1). In particular, the sequences surrounding Ser-8 and Ser-99/Ser-106 match the consensus sequences targeting phosphorylation by protein kinase CK2 and by proline-directed protein kinase(s) (MAP kinase and/or cyclin-dependent kinases), respectively. Actually mutation of Ser-99 to Ile significantly reduces Rev phosphorylation in cultured cells [11]; in addition, a reduced phosphorylation of Rev was observed by inserting the doublet DL instead of SD in the sequence (S_8DED) [11] whose serine conforms to the canonical consensus sequence of CK2 [15]. Here we show that indeed both CK2 and MAP kinase (and to a lesser extent cdc2) readily phosphorylate Rev *in vitro* with good kinetic parameters. We also demonstrate that CK2-directed phosphorylation of Rev *in vitro* and *in vivo* is dramatically

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FIG. 1. Amino acid sequence of HIV-1 Rev protein (HXB2 molecular clone). The one letter code for amino acids has been used. Potential sites for CK2 (S/T-X-X-E/D/Sp) and Pro-directed protein kinases (S/T-P) are in boldface. Underlining denotes the residues mutated in the present work.

increased in the presence of an amino-terminal mutation that is known to suppress Rev's functional activity [9] and would be expected to improve its susceptibility to CK2 [16].

MATERIALS AND METHODS

Plasmids. Eukaryotic expression plasmids pBsrev (encoding wild-type Rev) and pBsrev14-16EED (in which amino acids 14, 15 and 16 were changed from arg-thr-val to glu-glu-asp), and the prokaryotic expression plasmid pGST-rev (expressing wild-type Rev fused at the carboxy terminus of glutathione-S-transferase [GST]) have been described elsewhere [17]. To construct pLsRev8L, the coding portion of pBsrev was PCR-amplified using a sense primer that introduces a leucine codon at position 8, and an antisense primer corresponding to the 3′ end of the rev gene; the resulting fragment was ligated into the eukaryotic expression vector pLdK3pA [18]. pGST-Rev and pGST-Rev8L were constructed using the same PCR amplification strategy, with resulting fragments ligated into the vector pGEX2T [19], in frame with the GST gene. pGST-rev14-16EED was constructed by inserting the PCR-amplified coding portion of pBSRev14-16EED into pGEX2T, in frame with GST. Mutations were verified by DNA sequencing.

Purification of GST-Rev fusion proteins. E. coli of strain HB101 were transformed with the GST-Rev expression plasmids, grown to late-log phase, and then incubated for 2-4 hrs in the presence of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) to induce expression of the fusion proteins. The bacteria were then pelleted, frozen at $-80°C$, and lysed for 10 min at room temperature in 50 mM Tris, pH 8.0, containing 100 μg/ml lysozyme. Lysates were adjusted to 1% Triton X-100, passed through a 26-gauge needle, and clarified by centrifugation for 5 min at 17,500 $\times$ g. The supernatants were adjusted to 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride and the GST-Rev fusion proteins purified using glutathione-Sepharose followed by elution with 5 mM reduced glutathione, all as described [20].

In vitro phosphorylation of wild-type and mutant Rev proteins. Protein kinases CK1 and CK2 were purified as described in [21] from rat liver. MAP kinase p42 was purchased from Santa Cruz Biotechnology. $p34^{cdc2}$/cyclin B complex, purified from starfish oocytes, was a gift of Dr. Laurent Meijer (Roscoff, France). PKA was a commercial product of Sigma. Wild-type and mutant GST-Rev fusion proteins (3 μg) were incubated at 37°C for 10 min (unless differently indicated) in 50 mM Tris-HCl, pH 7.5 (25 μl final volume) containing 12 mM $MgCl_2$, 100 mM NaCl, 40 μM [γ^{32}P]ATP (specific radioactivity 1000-2000 cpm/pmol) and 10-50 mU of various protein kinases. NaCl was omitted when MAP kinase was the phosphorylating enzyme. The reaction was stopped by cooling in ice and followed by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, dried and scanned using a radioanalytical imaging system (Instant Imager, Canberra-Packard). Phosphate incorporated was quantified either directly from the autoradiograms or after excision of the bands and counting in a liquid scintillator.

Phosphopeptide mapping. Phosphorylated Rev protein was resolved by SDS-PAGE, transferred electrophoretically to nitrocellulose filters and localized by autoradiography. The excised bands were treated with polyvinylpyrrolidone and incubated with trypsin in 50 mM ammonium hydrogen carbonate, pH 8.9 at 30°C for 4 hrs [22]. The resulting peptides were separated on two-dimension-thin layer cellulose plates by electrophoresis at pH 8.9 in a buffer containing 50 mM ammonium hydrogen carbonate (40 min., 1000 V) followed by ascending chromatography in a buffer containing 25% piridine, 7.5% acetic acid, 37.5% butanol, and directly scanned using an Instant Imager.

In vivo phosphorylation of wild-type and mutant Rev proteins. The HeLa-derived cell line HLtat, which constitutively expresses HIV-1 Tat [23], was transfected [24] with pBsRev, pBsRev14-16EED or pLsRev8L. One day later, the cells were incubated for 1 hr in phosphate-free Dulbecco's modified Eagle's medium supplemented with 1% heat-inactivated fetal calf serum (phosphate-free DMEM), and radiolabeled for 3 hrs with 150 μCi ^{32}P/ml phosphate-free DMEM. The cells were rinsed once with PBS and then lysed in RIPA; resulting lysates were precleared with non-immune rabbit serum and protein A-Sepharose, and then immunoprecipitated with rabbit anti-sRev serum [17] and protein A-Sepharose. Immunoprecipitated proteins were separated by SDS-PAGE in 15% gels and electrotransferred to nitrocellulose; the ^{32}P incorporated into each Rev protein band was measured using an Instant Imager. To compare

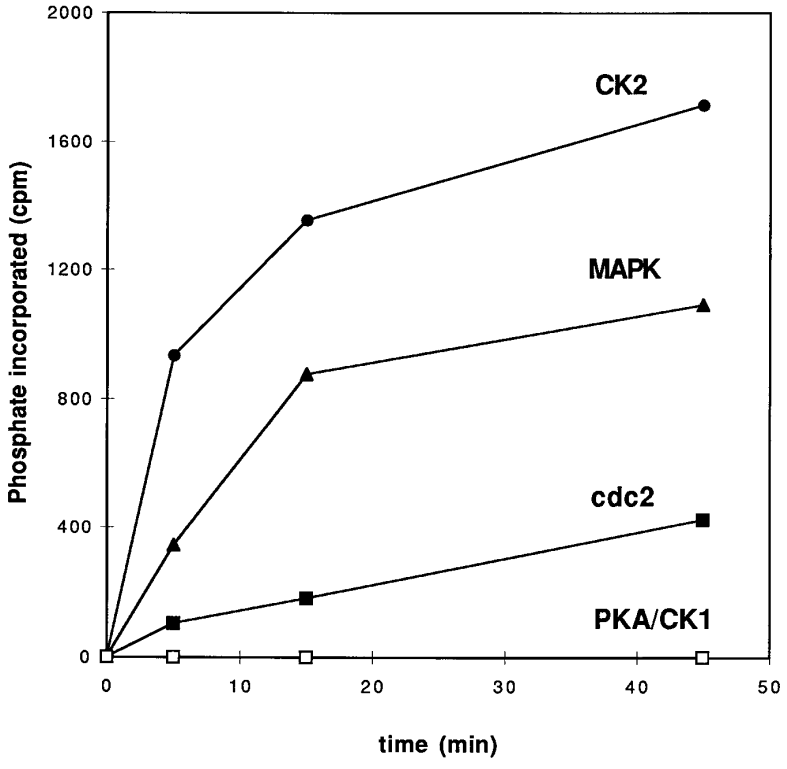

FIG. 2. Time course of phosphorylation of GST-Rev fusion protein by CK2, MAP kinase and cdc2 kinase. Phosphorylation conditions are described under Materials and Methods.

the amount of Rev protein present in each band, the membranes were probed with rabbit anti-sRev, developed using a chemiluminescent Western blot system (ECL, Amersham), and exposed to X-ray film. The Rev bands on the films were then quantitated by scanning densitometry.

RESULTS

Figure 2 presents a time course of *in vitro* phosphorylation of bacterially expressed, purified GST-Rev protein by an array of protein kinases. Results showed that Rev is readily phosphorylated by both protein kinase CK2 and MAP kinase. Phosphorylation by cdc2 is also detectable, while two other Ser/Thr protein kinases, PKA and CK1, are unable to phosphorylate Rev. Control assays performed using unfused GST protein showed that it is not detectably phosphorylated by CK2, MAP kinase or cdc2 (not shown). Double reciprocal plots from kinetic experiments (not shown) provided K_m values in the low μmolar range (2-5 μM) with CK2 and the two Pro-directed protein kinases. Surprisingly, phosphorylation of Rev by CK2 was not enhanced in the presence of polylysine, an activator of CK2 that is variably effective depending on the substrate [25], but instead, was slightly inhibited (not shown). Results of phosphoamino acid analyses confirmed that phosphoserine is the only product of phosphorylation by CK2, MAP kinase and cdc2 (not shown).

Following phosphorylation by CK2, MAP kinase or cdc2, Rev protein was subjected to tryptic digestion followed by phosphopeptide mapping. As shown in Figure 3, a single ^{32}P-labeled peptide is detected in a preparation of Rev labeled with CK2, while two radioactive spots (a major and a minor one), both distinct from the CK2 peptide, are obtained from Rev

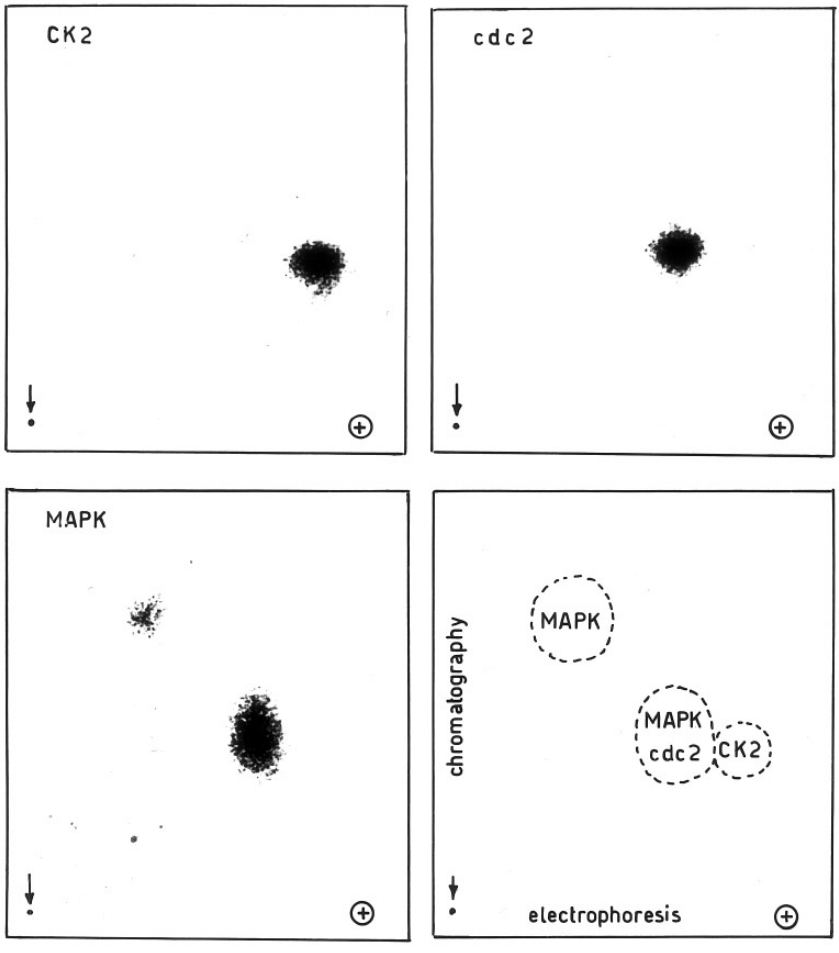

FIG. 3. Tryptic phosphopeptide mapping of Rev phosphorylated *in vitro* by CK2, cdc2, and MAP kinases. Tryptic digestion was performed as detailed under Materials and Methods. Only the autoradiogram is shown. The arrow indicates the position in which the hydrolysate was spotted onto the plate.

phosphorylated by MAP kinase. The main radioactive peptide detected in the digest of MAP kinase-radiolabeled Rev corresponds to the only product of Rev phosphorylated by cdc2 (Figure 3). These data indicate that: i) CK2 and MAP kinase phosphorylate distinct sites on the Rev protein; ii) a single site composed of one or more serines close to each other, is phosphorylated by CK2; iii) two sites are susceptible to MAP kinase phosphorylation, one of which overlaps with a target for cdc2 kinase.

In order to test whether Ser-8 is in fact phosphorylated by CK2, this residue, as well as Ser-5, which could become a ''secondary'' phosphorylation site upon phosphorylation of Ser-8 [26], were individually mutated to leucine. Mutant Rev 8L proved almost completely unable to undergo phosphorylation by CK2 (Figure 4); therefore, Ser-8 represents the major site of phosphorylation by this kinase. Mutant Rev 5L is still phosphorylated, although less readily than wild-type Rev. These data show that Ser-5 is likely to represent a secondary site, whose phosphorylation is directed by the presence of a phosphate group on Ser-8.

Substitution of Ser-5 or Ser-8 does not appear to seriously impair Rev's ability to transacti-

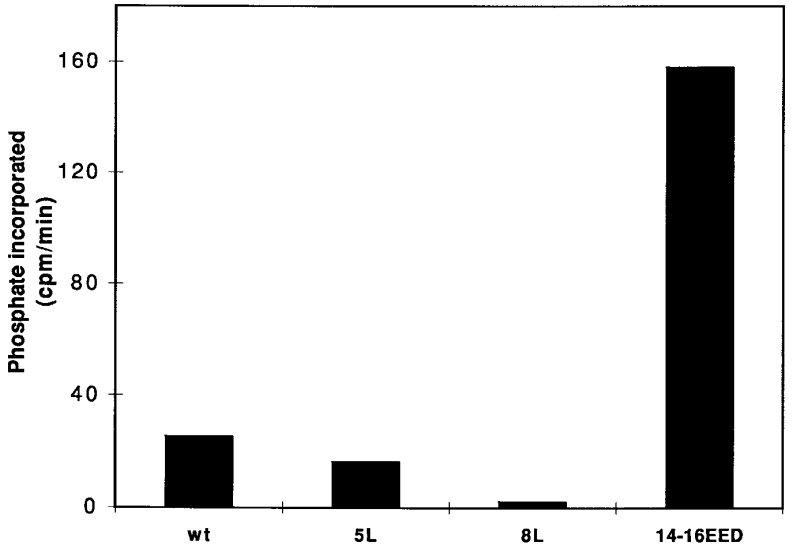

FIG. 4. *In vitro* phosphorylation of wild type and mutated GST-Rev proteins by protein kinase CK2. Phosphorylation conditions are described under Materials and Methods.

vate an RRE-containing mRNA [11]. However, introduction of three negatively charged amino acids (Glu-Glu-Asp) nearby at positions 14-16 (replacing Arg-Thr-Val) has been shown to disrupt its multimerization, RRE-binding, and functional activity (mutant Rev 14-16, ref. [10]). The identification of Ser-8 as the main phosphorylation site by CK2 prompted us to check whether the 14-16EED triple mutation may also influence phosphorylation of Ser-8/Ser-5 by CK2, whose activity is known to be enhanced by the presence of multiple acidic residues downstream from serine, even at rather remote positions [27]. As also shown in Figure 4, this mutation indeed dramatically increases phosphorylation by CK2. In contrast to CK2, MAP kinase phosphorylates Rev 14-16EED mutant at a level similar to wild type Rev (not shown).

Quantitative *in vivo* phosphorylation experiments using a HeLa cell line transiently expressing Rev proteins confirmed that the 14-16EED mutation causes a 25-30 fold (Table 1) increase

TABLE 1
In vivo Phosphorylation of Wild Type and Mutated Rev Proteins

	^{32}P/protein		Phosphorylation relative to Rev		Functional activity[a]
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	
Rev wild-type	6.48	1.57	1.0	1.0	+
Rev 14-16EED	168	47.6	25.9	30.3	−
Rev 8L	1.69	1.17	0.26	0.75	+

Phosphorylation conditions are described under Materials and Methods. ^{32}P/protein was calculated by dividing the ^{32}P cpm in the Rev band by the area under the peak of the same band detected after chemiluminescent Western blotting. Relative phosphorylation was calculated by dividing ^{32}P/protein value of mutant Rev by the ^{32}P/protein value of wild-type Rev. [a]The activity of Rev was determined in a cotransfection assay using the reporter plasmid pCgagA2pA as described [42].

in the phosphorylation of the protein, while mutation of Ser-8 to leucine suppresses phosphorylation, as suggested earlier using a similar mutant [11]. These results strongly indicate that Ser-8 serves as a site of CK2-directed phosphorylation *in vivo* as well as *in vitro*, and suggest that the loss of biological activity seen with the 14-16EED mutation may in part be due to hyperphosphorylation at Ser-8/Ser-5 by this protein kinase.

DISCUSSION

The observation that among the 10 seryl residues of Rev three conform to the consensus sequence of CK2 and two display the typical S-P motif recognized by Pro-directed kinases prompted us to investigate if these kinases are actually capable of phosphorylating Rev *in vitro.* Our data show that both CK2 and MAP kinase readily phosphorylate Rev with K_m values sufficiently low (2-5 μM) as to be considered physiologically relevant. In contrast, Rev does not appear to be a substrate of PKA or protein kinase CK1. The main phosphoacceptor site for CK2 has been identified by mutational experiments and shown to coincide with the expected Ser-8. Using phosphopeptide mapping we determined that at least two residues, both distinct from Ser-8, are phosphorylated by MAP kinase. Considering the compelling requirement of MAP kinase for a proline adjacent to the C terminal side of Ser/Thr [28], it is very likely that Ser-99 and Ser-106 represent the target for this enzyme. The implication of the other class of proline directed protein kinases, namely the cyclin dependent ones, is not supported by our data, which show that cdc2 is a much poorer phosphorylating agent of Rev compared to MAP kinase, an observation consistent with the fact that neither Ser-99 nor Ser-106 are followed by basic residue(s) which are required for efficient phosphorylation by cyclin dependent kinases but not by MAP kinase [28]. Interestingly, an early study of Rev phosphorylation identified Ser-99 (a probable MAP kinase site) and Ser-92 (showing no obvious consensus for a particular kinase) as the major sites of phosphorylation *in vivo,* and indicated that these residues could be substituted without loss of function [12].

From a mechanistic standpoint, the phosphorylation of Ser-8 by CK2 is notable in two respects, namely because it is not stimulated, but rather inhibited by polylysine, which normally increases CK2 activity toward most protein substrates (including the HIV-1 protein Vpu) and because it provides another example of ''hierarchical'' phosphorylation, in which primary phosphorylation of a residue generates the consensus for the subsequent phosphorylation of another nearby residue, in this case, Ser-5 [29]. In Rev, the phosphorylation of Ser-5, whose occurrence is documented by the reduced phosphorylation of Rev 5L mutant, is fully suppressed if the phosphorylation of Ser-8 (at the crucial n+3 position) is prevented by its mutation to leucine.

From a functional point of view, the phosphorylation of Rev by CK2, a pleiotropic and ubiquitous protein kinase hyperexpressed in tumors [30-33] is notable in several respects. Firstly, another protein expressed by HIV-1, Vpu, is phosphorylated by CK2 [35]; secondly, CK2 is also implicated in the phosphorylation of proteins expressed by other viruses (such as VP1 of polyomavirus, SV40-T antigen, ZEBRA protein of Epstein-Barr virus, E7 of human papilloma virus, gpI of varicella-zoster virus [35-39]) suggesting that it may play a general role in the processing/regulation of viral proteins by the host cell. Thirdly, the main target of CK2, Ser-8, is located upstream from a triplet of amino acids at positions 14-16, whose replacement by an acidic triplet both suppresses Rev activity [9] and appears to dramatically enhance the phosphorylation of Ser-8 by CK2. Although the phosphorylation of Rev *in vivo* by CK2 has not been incontrovertibly demonstrated, its occurrence is strongly indicated by the observed reduced *in vivo* phosphorylation of a Rev mutant in which Ser-8 was substituted by leucine and by hyperphosphorylation of the mutant Rev14-16EED, which also is specifically hyperphosphorylated *in vitro* by CK2 but not by MAP kinase. Although Ser-8 would appear

to represent a ''minor'' phosphorylation site *in vivo*, this does not in any way exclude the possible importance of this or other partially phosphorylated sites, which might appear to be underphosphorylated due to rapid dephosphorylation by protein phosphatases poorly active on the phosphorylated Ser-Pro motifs generated by proline-directed protein kinases [40, 41]. Consequently, CK2-phosphorylated Rev will become appreciable under conditions that either increase CK2 efficiency (for example in the presence of the 14-16EED mutation) or decrease protein phosphatase activity. Although early studies did not demonstrate that phosphorylation of Rev is necessary for the protein's functional activity [11,12], the opposite possibility, i. e. that phosphorylation at certain sites may serve to negatively regulate some aspect of the Rev response, has not yet been rigorously examined. It would be tempting in this connection to speculate that the loss of Rev activity induced by the 14-16EED mutation could be related to the increased phosphorylation of Ser-8 by CK2; the generation of new mutants in which the 14-16EED mutation is accompanied by that of Ser-8 and/or Ser-5 will be required in order to test the validity of this hypothesis.

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