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Nuclear export of late HIV-1 mRNAs occurs via a cellular protein export pathway

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ABSTRACT **The Rev protein of HIV-1 is essential for the nuclear export of incompletely spliced viral mRNAs. This action depends on the mutationally defined Rev activation domain, which both binds the nucleoporin-like human cellular cofactor Rab/hRIP and also functions as a nuclear export signal. Protein kinase inhibitor α (PKI) also contains a potent nuclear export signal. However, PKI plays no role in nuclear RNA export and instead induces the nuclear export of a specific protein target, the catalytic subunit of cAMP-dependent protein kinase. Here, it is demonstrated that the nuclear export signal of PKI not only binds the Rab/hRIP cofactor specifically but also can effectively substitute for the Rev activation domain in mediating the nuclear export of HIV-1 mRNAs. We conclude that HIV-1 Rev and PKI act through an identical nuclear export pathway and that Rev, rather than using a dedicated RNA export pathway, is instead acting as an adaptor that allows viral mRNAs to access a cellular protein export pathway.**

The Rev protein of HIV-1 is absolutely required for the expression of viral structural proteins and, hence, for viral replication (1–3). These late viral gene products are encoded by a class of incompletely spliced HIV-1 mRNAs that are, in the absence of Rev, sequestered in the infected cell nucleus. Rev induces the expression of these HIV-1 mRNAs by specifically activating their export from the nucleus (3–8). Specificity is conferred by a cis-acting RNA target site, termed the Rev response element (RRE) (4). Rev is believed to first bind to a single high-affinity target site within the RRE and to then extensively multimerize on the RRE (9–15). The resultant ribonucleoprotein complex then recruits a cellular cofactor(s) that mediates the entry of these viral RNA species into a preexisting nuclear RNA export pathway (3, 16–19).

At least two functional domains have been mapped within HIV-1 Rev (3). A domain closer to the amino terminal end includes an arginine-rich sequence that functions both as a specific RNA-binding domain and as a nuclear localization signal. This, in turn, is closely flanked by sequences that are essential for Rev multimer formation (12, 20–22). A second Rev domain closer to the carboxyl terminus, containing several critical leucine residues (see Fig. 1), is not required for interaction with the RRE but is essential for Rev function (12, 20, 22). This Rev "activation domain" was therefore proposed to act as a cofactor-binding domain (17, 20, 23, 24). Recently, this hypothesis has been confirmed with the demonstration that the Rev activation domain specifically binds to a nucleoporin-like human protein termed the Rev activation domain-binding protein (Rab) (19) or the human Rev interacting protein (16). Data arguing for a specific interaction between Rev and a nucleoporin-like cellular cofactor(s) have also been obtained in the yeast *Saccharomyces cerevisiae* (18). In human cells, Rab has been shown to significantly enhance Rev function when overexpressed (16, 19), strongly suggesting that Rab plays an important role in mediating Rev function.

In addition to mediating Rev cofactor binding, the Rev activation domain has also been shown to function as a nuclear export signal (NES) (17, 25). Analyses of available Rev mutants have suggested that activation domain function, Rab-binding ability, and NES function are tightly linked and are, indeed, likely to represent different aspects of a single biological activity (16, 17, 19, 25).

In addition to the Rev NES, a second NES has also recently been identified in human protein kinase inhibitor α (PKI) (25). The biological role of the 76-aa PKI protein is to bind the activated nuclear form of the catalytic subunit of cAMP-dependent protein kinase and to induce its export from the nucleus, thereby inhibiting its activity (26). PKI thus has no known role in any form of RNA export. Nevertheless, the NES found in PKI is somewhat similar to the Rev NES in that it also contains several critical leucine residues (see Fig. 1). Here, we demonstrate that the PKI NES specifically binds the cellular Rab cofactor and can efficiently substitute for the Rev activation domain in mediating the nuclear export and expression of late HIV-1 mRNAs. These data thus indicate that PKI, which normally mediates the nuclear export of a target protein, and Rev, which mediates nuclear export of target RNAs, are each acting via the same cellular nucleocytoplasmic transport pathway.

MATERIALS AND METHODS

Construction of Mammalian Expression Plasmids. All mammalian expression plasmids were based on pBC12/CMV, which contains the human cytomegalovirus immediate early promoter (20). Expression plasmids encoding wild-type (pcRev) and mutant (pcRevM10) forms of HIV-1 Rev have been described (20). The fusion protein expression plasmids pM9/PKI, pM9/P1, pM9/P6, and pM9/P11 were derived from the Rev mutant expression plasmid pcRevM9, which contains a unique inserted *Bgl* II site underlying Rev residues 67 and 68 (20). A cDNA encoding the wild-type human PKI sequence (amino acids 2–76) (25) was amplified by PCR using primers that inserted a 5′ *Bgl* II site in place of the normal translation initiation codon and that also inserted an *Xho* I site immediately 3′ to the PKI translation termination codon. The *PKI* gene was then inserted into pcRevM9 between the introduced *Bgl* II site and a unique *Xho* I site located 3′ to the Rev cDNA sequence, thus deleting sequences encoding amino acids 67–116 of HIV-1 Rev. The resultant pM9/PKI plasmid is predicted to encode a fusion protein consisting of amino acids 1–66 of Rev linked to amino acids 2–76 of human PKI. The pM9/P1, pM9/P6, and pM9/P11 expression plasmids are identical to pM9/PKI, except that they contain the previously described (25) two amino acid missense mutations of the PKI

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Abbreviations: NES, nuclear export signal; PKI, protein kinase inhibitor a; RRE, Rev response element; Rab, Rev activation domain-binding protein; CAT, chloramphenicol acetyltransferase.

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NES shown in Fig. 1. The sequences encoded by these fusion protein expression plasmids were each confirmed by DNA sequence analysis. The pDM128/CMV, pHIV-1ΔRev, and pgTat indicator constructs have been described (20, 21, 23).

Mammalian Cell Culture. COS cells were maintained as described (20) and were transfected using the DEAE-dextran procedure. Assays for Rev function in transfected COS cells using the indicator constructs pDM128/CMV, pHIV-1ΔRev, and pgTat have been described in detail (20, 21, 23). Western blot analysis of Rev protein expression levels in COS cells were done 48 hr after transfection, as previously described, using a rabbit polyclonal anti-Rev antiserum (20).

Yeast Expression Analysis. We have previously described the yeast expression plasmid pGAL4-Rev, which expresses a fusion protein consisting of the GAL4 DNA-binding domain linked to the HIV-1 Rev protein (19). Similarly, pGAL4-PKI was designed to express the GAL4 DNA-binding domain linked in-frame to amino acids 2–76 of human PKI. For this purpose, the wild-type *PKI* cDNA sequence was amplified by PCR using primers that inserted a unique *Eco*RI site at the translation initiation codon and inserted an *Xho* I site 3′ to the translation termination codon. The PKI open reading frame was then inserted into the polylinker *Eco*RI and *Sal* I sites present in the pGBT9 (Clontech) yeast expression plasmid, in-frame with the GAL4 DNA-binding domain. Derivatives of pGAL4-PKI that express fusion proteins containing the missense mutations indicated in Fig. 1 were constructed using standard recombinant DNA techniques.

To assess the ability of PKI to interact with the human Rab protein, the relevant GAL4 fusion protein expression plasmid was introduced into the Y190 yeast two-hybrid indicator strain (27) along with pVP16/Rab, which expresses a fusion protein consisting of the VP16 transcription activation domain linked to the full-length Rab open reading frame (19). The ability of PKI and Rev derivatives to interact with wild-type Rab was then assessed by quantifying the level of induction of the *lacZ* indicator gene present in the Y190 yeast strain, as previously described (19).

RESULTS

The activation domain of HIV-1 Rev is a short, leucine-rich sequence (23, 24, 28) that has previously been shown to specifically interact with a human cellular cofactor termed the Rab protein (19) or the human Rev interacting protein (16). To test whether the NES of PKI also shared this property, we examined the ability of wild-type PKI, or of selected mutant PKI proteins, to bind Rab using the yeast two-hybrid system (19, 29). Coexpression in the yeast Y190 indicator strain (27) of a VP16/Rab fusion protein (19) and of a fusion protein consisting of the GAL4 DNA-binding domain linked to PKI, resulted in the induction of levels of β-galactosidase activity that exceeded those seen with a similar GAL4–Rev fusion protein (Fig. 1; ref. 19). This induction, which was also observed with a fusion protein consisting of the GAL4 DNA-binding domain linked to a PKI mutant, termed P1, that retains a functional NES (25) (Fig. 1), was fully dependent on coexpression of the VP16/Rab fusion protein (data not shown). Mutations of the Rev activation domain, such as M10, that block Rev function have previously been shown to also block both Rab binding and NES function (16, 17, 19, 25). Similarly, two PKI missense mutations, termed P6 and P11, that have been shown to abrogate NES function (25) were found to also effectively block PKI binding by Rab (Fig. 1).

The NES of PKI can functionally replace the Rev activation domain. To test whether the PKI NES was indeed functionally equivalent to the Rev activation domain, we fused the amino-terminal 66 aa of HIV-1 Rev, which contain the complete Rev RNA binding/multimerization domain (12), to the PKI open reading frame. The resultant chimeric protein, which we term

		NES Function	RAB Binding
M10	- - - - - D L - - - -	–	<1
Rev (73-83)	L Q **L** P P **L** E R **L** T **L**	+	100
PKI (36-46)	E **L** A L K **L** A G **L** D **I**	+	151
P1	A - - - A - - - - - -	+	169
P6	- A - - - A - - - - -	–	<1
P11	- - - - - - - - - A - A	–	<1

FIG. 1. Alignment of the NESs of HIV-1 Rev and PKI. Leucines and an isoleucine known to be critical for Rev activation domain or PKI NES function are highlighted (23, 25). The ability of the indicated wild-type and mutant Rev and PKI sequences to function as NESs has been reported (17, 25), while their ability to bind Rab was determined using the yeast two-hybrid assay (19, 29). Sequences encoding wild-type or mutant HIV-1 Rev (amino acids 1–116) or human PKI (amino acids 2–76) were expressed fused to the GAL4 DNA-binding domain while the Rab protein was coexpressed as a fusion protein linked to the VP16 activation domain in the yeast indicator strain Y190 (27). Induced β-galactosidase levels were measured as described (19) and are given relative to wild-type Rev, which was arbitrarily set at 100. All fusion proteins were equivalently stable in yeast, as determined by Western blot analysis (data not shown).

M9/PKI, is predicted to retain the ability to bind the RRE RNA target but lacks all Rev-derived activation domain sequences (12, 23, 24, 28). Additional fusion protein expression plasmids, containing the PKI missense mutations listed in Fig. 1, were also generated.

The pDM128/CMV indicator plasmid contains a chloramphenicol acetyltransferase (CAT) gene, and the HIV-1 RRE, embedded in an HIV-1-derived intron (21, 23). CAT expression requires nuclear export of the unspliced form of the pDM128/CMV transcript and is therefore dependent on Rev function. In transfected COS cells, minimal CAT activity is seen in the absence of Rev (Fig. 2*A*) whereas expression of Rev results in an ≈20 fold increase in CAT activity. The RevM10 mutant, which lacks a functional activation domain/NES (17, 20, 25) (Fig. 1), is unable to enhance CAT expression. While the M9/PKI fusion protein, and the M9/P1 PKI mutant that retains a functional NES, also efficiently induced CAT activity, two mutants, termed M9/P6 and M9/P11, that lack a functional PKI NES (25) failed to activate CAT expression. This lack of activity did not result from the inefficient expression of these NES^- fusion proteins in that a Western blot analysis of their expression in transfected cells demonstrated levels that were at least as high as observed for the two active M9/PKI fusion proteins (Fig. 2*B*).

The HIV-1 Rev protein is required for the nucleocytoplasmic transport, and hence translation, of late HIV-1 mRNAs that encode the viral structural proteins, including Gag (1–6). Therefore, an HIV-1 provirus that lacks a functional Rev gene, termed pHIV-1ΔRev (20), gives rise to only minimal levels of p24 capsid protein expression after transfection into primate cells (Fig. 2*C*). Cotransfection of a wild-type Rev expression plasmid results in a robust activation of virion protein synthesis, but only if the Rev protein contains a functional activation domain (Fig. 2*C*). Cotransfection with the pHIV-1ΔRev indicator construct of plasmids expressing either the wild-type M9/PKI fusion or a mutant (M9/P1) that retains a functional NES also resulted in efficient rescue of HIV-1 structural protein expression. In contrast, similar Rev/PKI fusion proteins bearing a defective PKI NES were unable to activate HIV-1 capsid synthesis (Fig. 2*C*).

The data presented in Fig. 2*C* demonstrate that the PKI NES is able to functionally substitute for the essential Rev activation domain/NES in activating the expression of HIV-1 structural proteins. To confirm that this activation indeed reflected the cytoplasmic expression of unspliced HIV-1 mRNA species, we analyzed cytoplasmic viral RNA expression using a previously described S1 nuclease protection assay (4, 20, 23). The pgTat

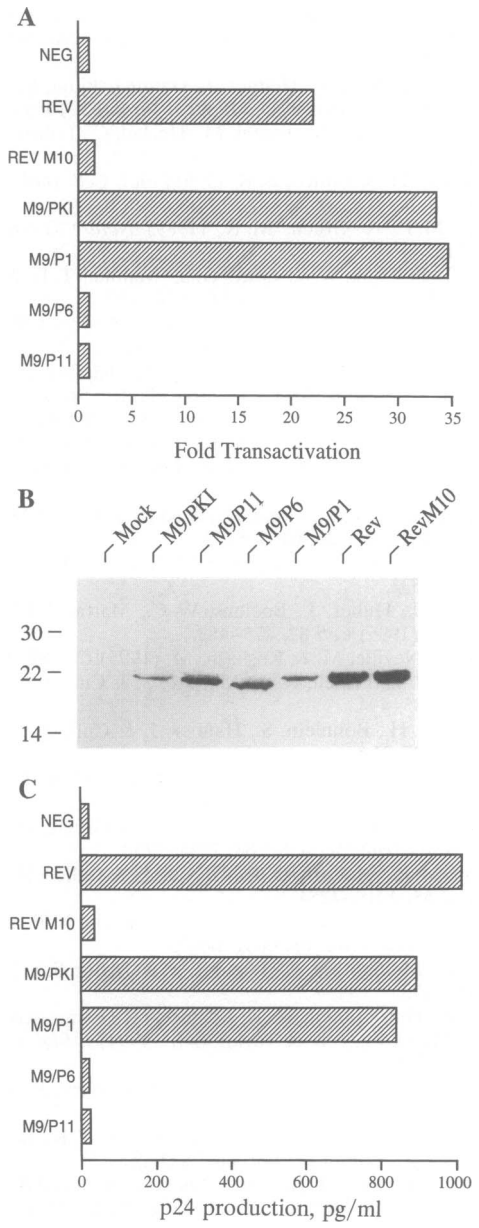

FIG. 2. The PKI NES can functionally replace the Rev activation domain. (*A*) Relative transactivation of the CAT gene present in the pDM128/CMV indicator plasmid (21, 23), induced by Rev or by the indicated Rev/PKI fusion proteins, was measured 48 hr after transfection into COS cells. (*B*) Western blot analysis of expression levels of the indicated Rev protein variants in transfected COS cell cultures. Relative mobility of protein molecular mass markers is given at left. (*C*) Ability of indicated Rev derivatives to induce expression of p24 capsid protein from the Rev-deficient HIV-1ΔRev provirus was determined ≈72 hr after transfection into COS cell cultures, as described (20).

indicator construct expresses the genomic *tat* gene, which consists of the two *tat* coding exons separated by an intron derived primarily from the viral envelope gene. In the absence of Rev, cells transfected with pgTat exclusively express a spliced form of the encoded *tat* mRNA in the cell cytoplasm (Fig. 3, lane 2). In contrast, coexpression of HIV-1 Rev induces the cytoplasmic expression of readily detectable levels of an unspliced *tat* mRNA (lane 3). This induction depends on the integrity of the Rev activation domain (lane 4). Importantly, unspliced cytoplasmic Tat mRNA expression was also efficiently induced by the M9/PKI fusion protein but not by the M9/P11 fusion protein lacking a functional PKI NES (lanes 5 and 6). Therefore, the PKI NES is clearly able to effectively substitute for the Rev activation domain/NES in inducing HIV-1 RNA export from the mammalian cell nucleus.

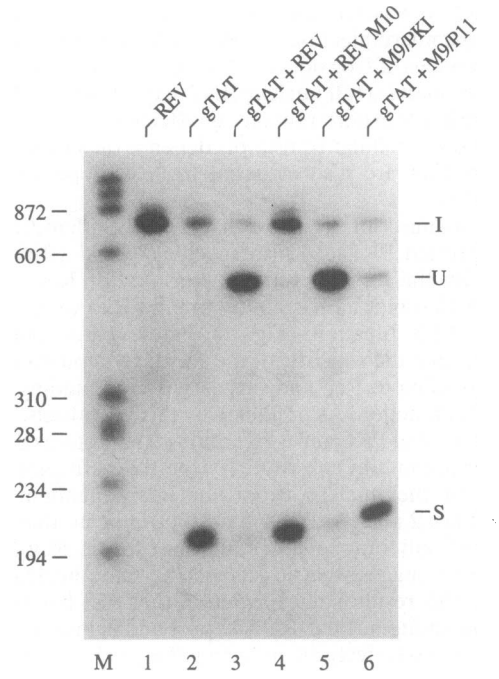

FIG. 3. The PKI NES can replace the Rev NES in inducing cytoplasmic expression of unspliced HIV-1 RNA. Quantitative S1 nuclease protection analysis of cytoplasmic RNA derived from COS cells transfected with the indicated plasmids was performed as described (4, 20, 23). The end-labeled 798-nt input (I) probe is designed to permit the quantitation of both unspliced (U) and spliced (S) cytoplasmic RNAs derived from the genomic HIV-1 *tat* gene present in the pgTat indicator plasmid. The unspliced *tat* transcript is predicted to rescue a 506-nt probe fragment, whereas the spliced *tat* mRNA is predicted to rescue a 202-nt fragment. M, marker lane containing end-labeled, *Hae*III-digested ϕX174 DNA.

DISCUSSION

The cellular pathways that mediate the export of RNA transcripts from the eukaryotic nucleus remain poorly understood (30). Competition experiments in frog oocytes that examined the ability of high levels of different RNAs to competitively inhibit the export of specific RNA classes have, however, strongly suggested that there are multiple RNA export pathways that are, at least in part, distinct (31). It has therefore been proposed that some steps in the nuclear export of tRNAs, spliceosomal small nuclear RNAs, cellular mRNAs, large rRNAs, and, possibly, 5S rRNAs require specific factors that are not involved in the nuclear export of other RNA classes (31). In large part, the identity of these factors has remained unclear, although evidence implicating a number of cellular proteins in the nuclear export of specific RNA classes has been presented (30).

In contrast to the situation with cellular RNAs, the nuclear export of late HIV-1 mRNAs is understood in significantly greater detail (3). Thus, the cis-acting RRE target for RNA export is fully defined, the viral Rev RNA export factor has been extensively characterized, and, most recently, evidence has been presented demonstrating that the Rev activation domain is not only a potent NES (17, 25) but also the binding site for a cellular nucleoporin-like cofactor that can markedly enhance Rev function *in vivo* (16, 18, 19). In human cells, this cofactor has been termed either Rab or human Rev interacting protein (16, 19). To this point, the relationship of the Rev-mediated RNA export pathway to the export pathways used by

cellular RNAs has remained unclear. However, data demonstrating that microinjection of a Rev activation domain peptide into the nucleus of frog oocytes can specifically block 5S rRNA and spliceosomal small nuclear RNA export, while tRNA and cellular mRNA export remains unaffected, suggest that the export of some cellular transcripts depends on a nuclear factor or factors that are also essential for Rev-dependent RNA export (17).

In this manuscript, we show that the NES identified in the cellular protein PKI (25) also specifically and efficiently interacts with the human Rab cofactor. As for Rev, missense mutations that block PKIs ability to bind Rab were also seen to block NES function (Fig. 1). These data support the hypothesis that the sole role of the Rev activation domain is to mediate binding to Rab and suggest that this binding, in turn, allows NES function. As predicted by this hypothesis, our data further show that PKI, and a PKI mutant that retains the ability to bind Rab, can fully substitute for the Rev activation domain in inducing the nuclear export of Rev-dependent mRNA species (Figs. 2 and 3). These data thus indicate that the PKI NES is not only functionally equivalent to the Rev NES but also strongly suggest that it acts via the same nuclear export pathway. This result is unexpected in that PKI has no known role in mediating nuclear RNA export and is instead believed to function exclusively in inducing the nuclear export of a target cellular regulatory protein (26). Therefore, it is apparent that the pathway used by HIV-1 Rev to facilitate viral RNA export from the nucleus is not a dedicated cellular RNA export pathway. Instead, Rev accesses a protein export pathway that readily permits target RNA species to be carried along. Currently, it is unclear whether this finding implies that cellular RNAs are also exported from the nucleus as a result of binding to proteins that contain NESs. However, as noted above, data have been presented suggesting that 5S rRNA and U1 small nuclear RNA use an RNA export pathway that is similar or identical to the one used by Rev-dependent mRNA species (17). It is therefore possible that there are no RNA-specific export pathways from the nucleus and that RNAs are instead invariably transported as passengers aboard one of, presumably, several protein export pathways.

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