Human Immunodeficiency Virus Type 1 Rev Is Required In Vivo for Binding of Poly(A)-Binding Protein to Rev-Dependent RNAs

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In the absence of Rev or the Rev-responsive element, the Rev-dependent human immunodeficiency virus type 1 (HIV-1) RNAs do not behave as mRNAs; rather, they exhibit nuclear defects in splicing and/or nuclear export and cytoplasmic defects in stability and translation. A translational initiation factor, eIF-5A, has recently been shown to bind specifically to the Rev activation domain. As the binding of poly(A)-binding protein 1 (PAB1) to the poly(A) tail of mRNAs is involved in both the stability and translation of cytoplasmic mRNAs, we investigated whether Rev might influence the association of PAB1 with cytoplasmic HIV-1 RNAs. Antibodies were generated against PAB1. We used these antibodies in an immunoprecipitation assay to detect specific binding of PAB1 to cytoplasmic mRNAs. We found that in the presence of Rev, PAB1 was associated with Rev-dependent and Rev-independent RNAs in the cytoplasm of transfected cells. However, in the absence of functional Rev, we found little or no PAB1 associated with Rev-dependent RNAs. These RNAs were capable of binding PAB1 in vitro. These results demonstrate that HIV-1 RNAs are defective in PAB1 association in the absence of Rev.

Human immunodeficiency virus type 1 (HIV-1) is able to regulate its gene expression through complex interactions of many different *cis*- and *trans*-acting viral and cellular factors. One of the viral products involved in this regulation, the Rev protein, has been implicated in the splicing, nuclear export, stability, and translation of HIV-1 RNAs (1, 3, 7, 8, 10-14, 16, 19, 20, 30). The observation of a particular effect seems to be dependent on the experimental system that is used. However, the net effect of the Rev protein in all systems that have been examined is to allow the production of viral structural proteins. The many different levels at which Rev exerts its effects may be interpreted as meaning either that Rev has many different mechanisms of action or that Rev has a single mechanism and that the different apparent functions of Rev are merely different system-specific manifestations of that function. The precise mechanism of Rev function has yet to be elucidated.

Although the direct binding of Rev to HIV-1 RNAs is required for Rev function, this binding is not sufficient for Rev function in vivo (2, 9, 15, 22, 25). These results, coupled with mutagenesis studies on the Rev protein, have elucidated a functional domain within Rev that must interact either directly or indirectly with some cellular factors (18, 21). In the absence of Rev, the Rev-dependent viral RNAs behave very differently from normal cellular mRNAs. Without Rev, these viral RNAs may be defective in their nuclear export, splicing, stability, and translational capacity. Elements within the viral RNAs, *cis*-acting repressor sequences (CRS), have been shown to confer this effect (7, 17, 26, 29). To date, the identities of the cellular

factors involved in these processes have remained elusive. It is apparent from these studies that the CRS in some way mask the viral RNA from being recognized as a normal cellular mRNA.

We have previously shown that in the absence of Rev or the Rev-responsive element (RRE), Rev-dependent HIV-1 RNAs accumulate in the cytoplasm of lymphoid cells (729 B cells) but are not translated (1). The cytoplasmic localization of these RNAs was demonstrated by using unspliced thymidine kinase pre-mRNA as a control for fractionation efficiency. Although the thymidine kinase pre-mRNA demonstrated a predominantly nuclear localization, more than 90% of the Rev-dependent HIV-1 RNAs were found in the cytoplasmic fraction. The sizes of these RNAs were unchanged by the absence of Rev, and these RNAs were polyadenylated, as judged by oligo(dT) selection (reference 1 and data not shown). The nontranslated cytoplasmic RNAs in these cells were present, not as free RNA but rather as a complex of 40S to 80S in size. Another group has also observed the formation of a similar 40S complex, in the absence of Rev, in fibroblast cells (8). The size of this complex indicated that these RNAs were not polysomal; however, these experiments did not resolve the identity of this complex. A Rev-dependent defect in the translation of cytoplasmic RNAs in Cos cells has also been reported (7, 16). In some of these experiments, in situ hybridization was used to validate the cytoplasmic nature of these RNAs. Therefore, there is an abundance of evidence that Rev has both a nuclear effect on splicing and export and a cytoplasmic effect on the translation of HIV-1 mRNAs. Translational initiation factor eIF-5A, which is thought to be involved in a late step in translational initiation, has been recently shown to bind the activation domain of Rev (27). eIF-5A has a nuclear and cytoplasmic subcellular localization and thus might be directly

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involved in both the nuclear and cytoplasmic effects of the Rev protein.

The mRNAs of eukaryotic cells are distinguished from other RNAs by the presence of a 3' stretch of polyadenylate residues [poly(A)]. Poly(A) has been shown to bind a specific cytoplasmic protein, poly(A)-binding protein 1 (PAB1) (6). This protein has been shown to be involved in mRNA translation at the level of 60S ribosomal subunit binding (23, 28). Additionally, PAB1 has been implicated in the stabilization of poly(A)-containing RNAs (5). Since in the absence of Rev, the Rev-dependent HIV-1 RNAs exhibits defects in RNA stability and translation, we investigated the potential involvement of PAB1 in cytoplasmic Rev function.

MATERIALS AND METHODS

Cell culture and transfection. The 729 B-cell line was used for transfections and maintained in Iscove's medium supplemented with 10% fetal calf serum. Transfections were performed by electroporation as described previously (3). Cells (2×10^7) were electroporated with 100 μ g of plasmid DNA and were harvested 24 to 48 h postelectroporation. The transfected DNA constructs have been previously described (1, 2).

Antisera and radioimmunoprecipitation. Polyclonal antisera were made in rabbits as described previously (33). The synthetic peptides NPVINPYQPPSY and CDENGSKGY GFV, derived from conserved regions within *Xenopus* PAB1, were used to generate antisera, referred to as 61925 and 62354, respectively. 729 B cells (2×10^7) were electroporated with DNA, and at 24 h posttransfection, the cells were pelleted and resuspended in 4 ml of methionine-cysteine-free RPMI 1640 medium supplemented with 20% dialyzed fetal calf serum. Trans 35 S label (400 μ Ci; ICN Biochemicals) was added, and the cells were allowed to incorporate label for 24 h. The cells were pelleted, washed with phosphate-buffered saline, and lysed in 1 ml of Nonidet P-40 (NP-40) lysis buffer (150 mM NaCl, 10 mM Tris [pH 8.0], 1.5 mM $MgCl_2$, 0.65% NP-40). The lysate was centrifuged at 14,000 rpm for 5 min at 4°C to remove debris. The supernatant was precleared with 20 μ l of a 50% slurry of protein G-Sepharose (Pharmacia) at 4°C for 1 h. The supernatant was then immunoprecipitated with 20 μ l of the indicated antisera with 20 μ l of protein G-Sepharose and separated on a 12.5% polyacrylamide gel with a 3.5% stacking gel. The gel was fixed in a solution of 30% methanol and 10% glacial acetic acid overnight, washed four times with water, and placed in a 1 M solution of sodium salicylate for 1 h. Fluorography was performed on the dried gel.

RNA immunoprecipitation and RNA PCR analysis. A cytoplasmic extract was prepared from transfected cells as described previously (3). The supernatant was centrifuged to remove debris, and 100 to 200 U of RNasin (Promega) was added. The extract was added to tubes containing 20 to 50 μ l of protein A-Sepharose or protein G-Sepharose (50% slurry) and either 20 μ l of anti-Rev antiserum, 5 to 20 μ l of anti-PAB1 antiserum 61925 or 62354, or 1 μ g of antialbumin antiserum. In addition, the tubes with anti-Rev and antialbumin also contained 5 μ g of rabbit anti-mouse immunoglobulin G. The samples were incubated for 1 to 16 h with rocking at 4°C or room temperature. The immunoprecipitate was prepared by low-speed centrifugation and four washes of the pellet with NP-40 lysis buffer. RNA was prepared as previously described (2). RNA PCR analysis was performed as described previously (1–3), with the following modifications. PCR amplification was performed with a Perkin-Elmer Cetus GeneAmp PCR System 9600 programmed for 30 s of denaturation at 94°C and 2 min of polymerization at 65°C. The number of cycles for each

oligonucleotide pair includes 25 cycles for *env/vpu2*, 20 cycles for actin and *tat/rev*, and 10 or 15 cycles for U1. These conditions produce a quantitative signal for each RNA (reference 1 to 3 and data not shown). The nucleotide sequences for the U1 oligonucleotide primers are as follows: U1+, 5'-CCTGGCAGGGGAGATACCAC-3'; and U1-, 5'-CACTAC CACAAATTATGCAG-3'.

Production of recombinant human PAB1. Expression of human PAB1-maltose-binding protein (MBP) fusion protein was performed as follows. By using 1 μ g of cytoplasmic RNA from 729 B cells, cDNA was prepared with a PAB1-specific antisense oligonucleotide primer (5'-AACGGATCCTTAAACAGTTGGAACACCGG-3'). Conditions were as recommended in the GeneAmp RNA PCR kit (Perkin-Elmer Cetus). A 1,911-bp human PAB1-specific PCR product was generated by using the above antisense primer with a PAB1-specific sense oligonucleotide primer (5'-CGCGGATCCATGAACCCAGTGCCCCCAGC-3'). PCR conditions were as previously described (2). The PCR product was cut with *Bam*HI and cloned into pMAL-c2 (New England Biolabs). The plasmid was transformed into *Escherichia coli* TB1 and protein production was induced as instructed by the manufacturer. The construct should direct synthesis of an approximately 110-kDa PAB1-MBP fusion protein. Lysates from induced and uninduced cells were analyzed by Western blotting (immunoblotting) using anti-PAB1 antiserum 61925 (1:2,000) as instructed by the manufacturer.

PAB1 binding assay. PAB1 binding assays were performed essentially as described previously (24), with the following modifications. The first incubation (1 h at 4°C) included 20 μ l of anti-PAB1 antiserum 61925 in 400 μ l of NP-40 lysis buffer with 20 μ l of protein G-Sepharose (50% slurry). The beads were then washed four times with NP-40 lysis buffer. The second incubation (1 h at 4°C) included 100 ng of purified *Xenopus* PAB1 (32) in 400 μ l of NP-40 lysis buffer. The beads were then washed four times with binding buffer (100 mM NaCl, 30 mM Tris [pH 8.0], 1 mM EDTA, 15 mM 2-mercaptoethanol). The third incubation included 100 μ g of bovine serum albumin, 100 μ g of tRNA, and 200 U of RNasin (Promega) in 400 μ l of binding buffer for 30 min at 25°C. RNA ($\sim 10^6$ cell equivalents) was then added, and the fourth incubation continued for ~ 16 h at 25°C. The beads were washed four times with binding buffer. RNA was prepared from the immunosupernatants and the immunoprecipitates and subjected to RNA PCR analysis. Blocking was performed by the addition of 1 μ g of peptide to the first incubation or 100 μ g of poly(A) to the third incubation.

RESULTS

Antibodies against PAB1. We have previously demonstrated that in lymphoid cells (729 B cells), Rev has no effect on the cytoplasmic accumulation of HIV-1 singly spliced RRE-containing RNAs (1). We had previously used these cells since they were lymphoid in origin and should therefore be more closely related to the major natural cell type infected by HIV-1, T cells, than the routinely used fibroblast cell lines. Additionally, we had developed a transfection and RNA PCR analysis procedure adequate for the analysis of RNA expression in this cell line. Without Rev, the singly spliced, RRE-containing RNAs were defective in polysome formation and were not translated (1). As PAB1 is required for the completion of translational initiation, we wished to assess the involvement of PAB1 in HIV-1 Rev function, using an immunoprecipitation assay. We attempted to generate antibodies against PAB1. PAB1 is highly conserved among species, with 96% amino acid

FIG. 1. Antisera against human PAB1. (A) A crude cytoplasmic extract was prepared from lymphoid cells metabolically labeled with [35 S]methionine-cysteine. Two antisera generated against peptides corresponding to conserved regions of PAB1, as well as a previously described antiserum generated against a PAB1 fusion protein, were used to attempt to immunoprecipitate human PAB1. The band apparent in all lanes at ~69 kDa was shown to represent albumin by specific immunoprecipitation. (B) Protein expression from bacterial cells containing the human PAB1-MBP construct was induced with IPTG. The negative control bacterial cells did not contain this construct. Cell lysates were resolved on a 10% polyacrylamide gel and transferred to nitrocellulose. Immunoblots were performed with anti-PAB1 antiserum 61925 and the ECL detection system (Amersham). Sizes are indicated in kilodaltons.

homology between *Xenopus* and human PAB1. The antisera were generated by inoculation of rabbits with peptides corresponding to specific regions of PAB1. These antisera were tested for the ability to immunoprecipitate PAB1 from a human lymphoid cell line (729 B cells). The conditions used for this immunoprecipitation were similar to those used previously to detect the *in vivo* binding of HIV-1 Rev to the Rev-dependent RNAs (8). We hoped that these conditions were mild enough not to disrupt RNA-protein binding. This was important because we hoped to use the antiserum to immunoprecipitate PAB1 bound to mRNAs. The results are shown in Figure 1A. Under these conditions, specific immunoprecipitation of human PAB1 was seen with only one of the antisera, designated 61925. The size of the immunoprecipitated protein was approximately 70 kDa, consistent with the predicted molecular weight for this protein. These results showed that we were able to generate antibodies directed against human PAB1. The antibodies could be used to specifically immunoprecipitate PAB1 under conditions which should allow the analysis of PAB1-RNA interactions.

We further assessed the specificity of antiserum 61925 for human PAB1 by performing an immunoblot on human PAB1 expressed in bacteria. The results can be seen in Fig. 1B. Antiserum 61925 detected a protein of approximately 110 kDa, consistent with the predicted size of the MBP-PAB1 fusion protein. This protein was detected only in the bacterial lysate from the cells containing the human PAB1-MBP construct and only when these cells were induced with isopropylthiogalactopyranoside (IPTG). These results demonstrated the specificity of the anti-PAB1 antiserum 61925.

Binding of PAB1 to HIV-1 RNAs in the presence of Rev. We wished to determine if PAB1 was associated with the cytoplasmic HIV-1 RNAs in the presence of functional Rev protein. We expected that both the Rev-dependent and Rev-independent RNAs would be associated with PAB1, since both classes

FIG. 2. PAB1 is bound to HIV-1 mRNAs *in vivo*. A cytoplasmic extract from cells transfected with pYK_{JR-CSF}, a wild-type HIV-1 proviral construct, was aliquoted into two samples and immunoprecipitated in duplicate with the indicated antisera. RNA was prepared from the immunoprecipitates and analyzed by RNA PCR specific for the indicated RNAs.

are translated in the presence of Rev. To examine this association, we established an immunoprecipitation assay which used the anti-PAB1 antiserum to immunoprecipitate mRNAs through their association with PAB1. A cytoplasmic lysate was prepared from lymphoid cells (729 B cells) transfected with an infectious proviral construct of HIV-1_{JR-CSF}. This lysate was immunoprecipitated in duplicate with anti-PAB1 or control antiserum (anti-albumin). RNA was prepared from the immunoprecipitates and examined by RNA PCR for specific RNAs. The results are shown in Fig. 2. There was little or no difference in the level of U1 RNA, a nonpolyadenylated RNA, in the immunoprecipitations using either antiserum. In contrast, examination of β-actin RNA showed high levels of specific immunoprecipitation with the anti-PAB1 antiserum. Therefore, this antiserum could be used to specifically immunoprecipitate polyadenylated RNAs, and not nonpolyadenylated RNAs, through their association with PAB1. The Rev-independent *tat/rev* and Rev-dependent *env/vpu* 2 RNAs also demonstrated high levels of specific immunoprecipitation with the anti-PAB1 antiserum. The similarity in the signals obtained from the duplicate samples demonstrated the reproducibility of this immunoprecipitation procedure. These results demonstrated that *in vivo*, PAB1 was associated with both the Rev-dependent and Rev-independent RNAs in the presence of Rev. This antiserum provided us with a tool with which to analyze the effect of the HIV-1 Rev protein on the association of PAB1 with HIV-1 RNAs.

Binding of PAB1 to HIV-1 RNAs in the absence of Rev. To determine if Rev was necessary for the association of the Rev-dependent RNAs with PAB1, we examined the cytoplasmic lysate from lymphoid cells (729 B cells) transfected with the nonfunctional Rev mutant TDBg (2) for PAB1 association. We have previously shown that in lymphoid cells (729 B cells), Rev has no effect on the cytoplasmic accumulation of the singly spliced HIV-1 RNAs (1). We have also shown that the cytoplasmic fraction from cells fractionated by our procedure contains mainly cytoplasmic RNAs (1). This proviral construct has a single amino acid change of leucine to serine at amino acid 81 of the Rev protein and, although defective for Rev function, retains *in vivo* RNA binding of the Rev protein (2).

FIG. 3. Rev is necessary for the association of HIV-1 RNAs with PAB1. (A) A cytoplasmic extract from cells transfected with construct TDBg, a proviral construct which produces a nonfunctional Rev protein which is still able to bind to the RRE-containing RNAs, was immunoprecipitated as in Fig. 2 with the indicated antisera. RNA was prepared from the immunoprecipitates and analyzed by RNA PCR specific for the indicated RNAs. (B) A cytoplasmic extract from cells transfected with construct TDBg was immunoprecipitated with the indicated antisera in the presence (+) or absence (-) of 1 μ g of the PAB1-specific peptide against which antiserum 61925 was generated. RNA was prepared from the immunoprecipitates and analyzed by RNA PCR specific for the indicated RNAs.

Immunoprecipitations similar to those shown in Fig. 2 were performed. An additional immunoprecipitation with anti-Rev antibodies was performed as a positive control for binding to the Rev-dependent RNAs. RNA was prepared from the immunoprecipitates and assayed by RNA PCR. The results are shown in Fig. 3A. The level of U1 RNA immunoprecipitated was not significantly different in any of the immunoprecipitations with any of the antisera. In agreement with the immunoprecipitations performed with the wild-type proviral construct (Fig. 2), high levels of the Rev-independent β -actin and *tat/rev* RNAs were specifically immunoprecipitated with the anti-PAB1 antiserum in the absence of functional Rev. Interestingly, we were able to specifically immunoprecipitate little or none of the Rev-dependent RNAs (*env/vpu2*, *gag/pol*, *vpr*, and *vif*) with the anti-PAB1 antiserum, although they were readily immunoprecipitated by anti-Rev antibodies as previously reported (2). The results demonstrated that only in the presence of functional Rev protein were the cytoplasmic Rev-dependent RNAs able to associate normally with PAB1 in vivo.

The specific immunoprecipitation of translated mRNAs by anti-PAB1 antiserum 61925 was reproducibly seen in many experiments. In the same experiments, a lack of PAB1 association with the Rev-dependent RNAs (in the absence of functional Rev) was reproducibly seen. The majority of the selective immunoprecipitation of translated mRNA (*tat/rev*) by anti-PAB1 antibodies could be blocked by addition of the PAB1-specific peptide against which the antibodies were made (Fig. 3B), further demonstrating the specificity of the immunoprecipitation procedure. In this experiment, as with other experiments, the nontranslated *env/vpu2* and U1 RNAs were

FIG. 4. In vitro binding of PAB1 to HIV-1 RNAs. Purified RNA was prepared from cytoplasmic extracts of cells transfected with the Rev mutant TDBg. This RNA was incubated with purified *Xenopus* PAB1, and binding of PAB1 to RNA was detected by immunoprecipitation. RNA was prepared from the immunoprecipitates (PPT) and supernatants (SUPT) and subjected to RNA PCR analysis for the indicated RNAs. Where indicated, the specific binding of PAB1 was blocked either by addition of 1 μ g of the peptide against which the antiserum was made or by addition of 100 μ g of poly(A).

not specifically immunoprecipitated and were not affected by the presence or absence of the PAB1-specific peptide.

In vitro binding of PAB1 to HIV-1 RNAs. We wished to determine if the lack of association of PAB1 with the Rev-dependent RNAs, in the absence of Rev, was due to some inherent defect in the RNAs. We purified RNA from a cytoplasmic extract of cells transfected with the Rev mutant TDBg and used an in vitro binding assay to examine whether the Rev-dependent RNAs were capable of binding PAB1. Purified *Xenopus* PAB1 was used together with the anti-PAB1 antiserum in this assay system. PAB1 was incubated with the antiserum, which was previously bound to protein G-Sepharose. The bound PAB1 was then incubated with the purified RNA. As a control, either the peptide against which the antiserum was made or poly(A) was added to specifically inhibit the binding assay. RNA was prepared from the supernatant and precipitate and subjected to RNA PCR for specific RNAs. The results are shown in Fig. 4. Little or none of the nonpolyadenylated U1 RNA was bound by PAB1, demonstrating the specificity of the system. The Rev-dependent *vif*, *vpr*, and *env/vpu2* RNAs as well as the Rev-independent *tat/rev* and actin RNAs were all capable of binding to *Xenopus* PAB1. Addition of either the peptide against which the antiserum was generated or poly(A) blocked this binding, demonstrating the specificity of this interaction. These results indicated that in the absence of Rev, the Rev-dependent RNAs were capable of binding PAB1, although they were not bound by PAB1 in vivo. Therefore, the lack of PAB1 association in vivo with the Rev-dependent RNAs was not due to some inherent defect in the RNAs.

DISCUSSION

We have previously demonstrated that in lymphoid cells (729 B cells), Rev has no effect on the cytoplasmic accumulation of the singly spliced HIV-1 RNAs (1). In the absence of Rev or the RRE, these Rev-dependent cytoplasmic RNAs are defective in polysome formation and are found in a 40S to 80S complex that is sensitive to EDTA (1). In this report, we extend the previous observations and find that these Rev-dependent cytoplasmic RNAs are not associated *in vivo* with PAB1 in the absence of a functional Rev protein. However, they are capable of binding PAB1 *in vitro*.

The lack of PAB1 binding to these RNAs might lead to a decrease in the stability of these RNAs (5). A previous report has shown that Rev does indeed exert an effect on the stability of HIV-1 RNAs (12). PAB1 has also been shown to be necessary for the transition from a preinitiation complex to a monosome by the joining of the 60S ribosomal subunit (28). Consequently, the Rev-dependent RNAs should be unable to complete this step in translational initiation. However, it is also possible that these RNAs are blocked at some prior step. Several previous reports have shown that Rev is necessary for the translation of cytoplasmic Rev-dependent RNAs (1, 7, 8, 16). The size and EDTA sensitivity of the cytoplasmic complex formed in the absence of Rev are consistent with those of a preinitiation complex (1, 8). This complex might contain the Rev-dependent RNA, the 40S ribosomal subunit, and associated initiation factors (for a review, see reference 31). Alternatively, this complex may represent some other translationally arrested cellular complex. Recently, the direct binding of Rev to a translational initiation factor, eIF-5A, has been reported (27). In this report, it was shown that eIF-5A has both nuclear and cytoplasmic subcellular localization. Thus, it seems possible that eIF-5A may be involved in the nuclear and cytoplasmic effects of Rev. eIF-5A appears to be involved in a late step of translational initiation (4). As such, it seems plausible that eIF-5A may be involved in the interaction of PAB1 with the mRNA or the large ribosomal subunit. Further work will be needed to address these possibilities.

The lack of functional Rev leads to a defect in the association of PAB1 with the poly(A) tail of the Rev-dependent RNAs. These RNAs are in some way masked from recognition by PAB1 and the translational machinery. Elements within the viral genome, termed CRS elements, are presumably responsible for this masking of the Rev-dependent RNAs. We hypothesize that Rev might function either directly or indirectly to allow the unmasking of the poly(A) tail and allow its normal association with PAB1. It seems likely that eIF-5A would be involved directly in this process. The binding of PAB1 would promote the stability and translation of these RNAs. We believe that the data presented in this report and elsewhere are consistent with such a theory of Rev function; however, further work is required to determine how the presence of the CRS leads to the defect in PAB1 binding and the precise mechanism by which Rev is able to restore PAB1 binding to these RNAs. Additionally, the results presented in this report address only the cytoplasmic role of Rev in RNA stability and translation. They do not address the function of Rev in the nucleus in relation to its role in nuclear export and splicing. As very little is known concerning the role of nuclear PABs, it is difficult to say whether these proteins might be involved in nuclear Rev function.

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