In Vitro Interaction between Human Immunodeficiency Virus Type 1 Rev Protein and Splicing Factor ASF/SF2-associated Protein, p32*

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Continuous replication of human immunodeficiency virus type 1 (HIV-1) expresses at least five proteins, Gag, Pol, Env, Tat, and Rev, which are absolutely essential for viral replication. Gag, Pol, and Env proteins, which are associated with the virion particle, are encoded by unspliced and singly spliced mRNA species, characteristic for the late stage of the viral gene expression. Tat and Rev, translated from multiply spliced HIV-1 mRNAs characteristic for the early stage of the gene expression, have regulatory roles in the viral life cycle. Although Tat and Rev share some structural features, their functions are distinct. Tat binds to the transactivating region, TAR, located in the 5′-end of the viral transcript and up-regulates the viral transcription several hundred-fold. Rev acts at the posttranscriptional level and stimulates the cytoplasmic appearance of unspliced and singly spliced viral mRNAs (for reviews, see Refs. 1–3). Thus, Rev activity partially suppresses its own synthesis and shifts the HIV-1 replication cycle from the early to the late phase. The specificity of Rev activity is mediated through direct binding to an RNA element, the RRE, which is a cis-component of the unspliced and singly spliced mRNAs (4–6). The Rev protein is quite small (116 amino acids), and two main functional domains have been defined by mutational analysis. A cluster of leucine residues around positions 78–83 constitutes the nuclear export signal (7–10), which interacts directly with nuclear transport proteins involved in RNA export (11–13). A highly basic region at positions 34–50 is responsible for specific RNA binding (14–18). Nuclear localization, and contributes to the oligomerization process (18–20). This domain has also been shown to inhibit the splicing of RRE-containing mRNAs in vitro (21, 22). It was recently shown in a yeast two-hybrid screen that the murine protein, YL2, interacts with the basic domain of Rev and that overexpression of YL2 potentiates Rev function in vivo (23). The human homologue of YL2 is the p32 protein, which copurifies with the essential splicing factor ASF/SF2 (24). In this report, we investigate the interaction of p32 with Rev and test its function in a Rev-dependent in vitro splicing assay. We demonstrate that Rev interacts strongly with p32 in vitro and map the binding site by protein footprinting. Together, our data suggest that p32 functions as a mediator of Rev activity in RNA splicing.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The full-length p32 clone was a gift from Henrik Leffers and has been described previously (25). Plasmids used for expression of pro-p32, containing amino acids 1–282 (pGEX-ZTK-pro-p32 and pGEX-GTH-pro-p32), and the processed form of p32, containing amino acids 74–282 (pGEX-ZTK-p32 and pGEX-GTH-p32), were constructed by inserting the corresponding open reading frames, prepared by polymerase chain reaction synthesis, between the BamH I and EcoRI sites of the pGEX-ZTK vector (Pharmacia Biotech Inc.) and the pGEX-GTH vector (26). Expression of these constructs in bacteria, e.g., for the processed version, gave rise to GST-T-HMK-p32 (denoted GST-p32) and GST-T-p32-HMK (denoted GST-p32-K) types of proteins, respectively. GST denotes a glutathione S-transferase fusion, enabling rapid purification on immobilized glutathione-Sepharose 4B beads (Pharmacia), T marks the position of a cleavage site for thrombin protease, and HMK denotes the recognition site for the catalytic subunit of cAMP-dependent heart muscle kinase, enabling specific radio-labeling of the recombinant protein. The polymerase chain reaction fragments encoding pro-p32 and p32 were obtained using upstream primers GAGGATCCATGTCTCGCTCTGTGTC and GAGGATCCCTGACACCGAGGGAG, respectively, and a common downstream primer, GAGAATCTCTGTGACTACACCT (restriction sites for cloning are underlined). The expression plasmid for His-tagged Rev protein was a gift from Alan W. Cochrane and produces Rev preceded by 6 histidine residues (37).

Expression and Labeling of Proteins—The pro-p32 protein and the processed form of p32 were expressed in Escherichia coli strain AD202 (28), purified, and labeled at the HMK site as described in Ref. 26. p32 was eluted as a GST fusion protein (GST-p32 or GST-p32-K) by reduced glutathione or cleaved off the column by thrombin, yielding p32 attached to an HMK site at the C terminus (p32-K), followed by dialysis against p32 storage buffer (10 mM Tris/HCl, pH 7.5, 100 mM NaCl).
Expression and purification of His-tagged and wild type Rev proteins were performed as described previously (27, 29). His-tagged Rev was purified in a denatured form and renatured by dialysis at 4 °C against Rev storage buffer (50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA). The activity of Rev was assessed by RNA band shift assays described elsewhere in this section. We observed no difference in RNA binding activity between wild-type and His-tagged and wild-type Rev proteins (data not shown). Generally, His-tagged Rev was used in all experiments, except from the footprinting analysis, in which wild type Rev was used. Rev-(34–50) peptide was made as described previously (21).

Co-precipitation Assay—The Rev-p32 co-precipitation assay was performed by mixing 1 μl of p32 storage buffer, containing 2 μg GST-p32 (as a control), 1 μl of Rev storage buffer, containing 0.3 μg of Rev protein, with 4% native polyacrylamide gels containing GST-p32 was incubated with Rev prior to the addition of the RNA. The control) was added to each of the Rev titrations, and the incubation was continued with rocking for 1 h at 4 °C. Beads were washed five times in co-precipitation buffer without bovine serum albumin, and retained proteins were released by incubating the beads in 10 μl of 1 × SDS loading buffer (58 mM Tris/HCl, pH 6.8, 6% glycerol, 1.7% SDS, 0.0025% Serva Blue W, 0.8% β-mercaptoethanol) at 95°C for 5 min. The samples were loaded on 0.1% SDS, 16.3% polyacrylamide gels (7% stacking gels), and proteins were visualized by Coomassie staining.

The co-precipitation assays among GST-p32, Rev, and radioactive RRE RNA or IIB RNA were performed by mixing 1 μl of p32 storage buffer, containing 100–600 ng of GST-p32 (or 600 ng of GST as a control) and 1 μl of Rev storage buffer, containing 0–300 ng of Rev (or 0–100 ng of Rev-(34–50)) with 4 × 10^10 cpm (approximately 2 ng) of body-labeled, renatured RRE or IIB RNA in 8 μl of Rev binding buffer (10 mM HEPES/KOH, pH 7.9, 100 mM KC1, 2 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5 units/ml RNasin (Promega), 50 ng/μl E. coli tRNA) containing 0.2% Tween 20 and 0.2 μg/μl bovine serum albumin, followed by incubation at 4 °C for 1 h. Twenty μl of a 50% slurry of glutathione-Sepharose beads equilibrated in co-precipitation buffer, were added to each binding reaction, and incubation continued with rocking at 4 °C for 1 h. Beads were washed five times in Rev binding buffer (with Tween 20 and without bovine serum albumin) and then resuspended in 80% formamide, incubated at 95°C for 5 min, and fractionated on a 6% polyacrylamide gel containing 8 μl of Rev Tris borate, 2.5% SDS, and 1.5 mM EDTA. Gels were autoradiographed and quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software.

RESULTS

Pro-p32 Is Processed in Bacteria—The p32 protein is synthesized as a 282-amino acid-long pro-protein that is post-translationally processed in human cells by removal of the N-terminal 73 amino acids to form the 209-amino acid-long mature p32 protein (25). Both forms of the protein were expressed as fusion proteins, containing the GST tag at the N terminus. In addition, the constructs contained the recognition sequence for the catalytic subunit of cAMP-dependent HMK, either between the GST-tag and p32 (GST-p32), or at the C terminus of the p32 protein (GST-p32-K). The advantage of the latter construct is that only full-length affinity-purified protein becomes labeled, which is particularly important for protein mobility shift analysis and footprinting applications (26). The pro-p32 fusion proteins were highly unstable when expressed in bacteria. A major band co-migrating with the processed form of the recombinant p32 protein suggested that the precursor protein was processed in bacteria near or at the same site as observed in human cells (data not shown). In all our investigations, only the constructs expressing the processed form of p32 (amino acids 74–282) were used.

p32 Interacts Strongly with Rev in Vitro—To study the interaction between p32 and Rev we investigated the ability of Rev to co-precipitate with GST-p32 protein on glutathione beads in the presence of increasing concentrations of NaCl or the nonionic detergent, Tween 20 (Fig. 1A). High ionic strength binding and washing buffers preferably destabilize ionic interactions, whereas increased concentrations of nonionic detergents destabilize hydrophobic interactions. Rev was specifically and quantitatively co-purified with p32 on glutathione beads under stringent conditions (Fig. 1A, lanes 8–17). The p32-Rev interaction remained fully stable in binding and washing solutions containing 500 mM NaCl and in the highest tested Tween 20 concentration at 5% (Fig. 1A, lanes 10 and 17). At 750 mM NaCl, a slight decline in retained Rev was observed, and at 1 mM NaCl most of the Rev was removed from GST-p32 (Fig. 1A, lanes 11 and 12). GST-p32 remained stably associated with the glutathione beads at all conditions (Fig. 1A, lanes 8–17). In control reactions, in which GST-p32 was omitted or replaced with GST alone, only negligible amounts of Rev were retained on the beads after washing in a buffer containing 150 mM NaCl (Fig. 1A, lanes 6 and 7).

The interaction between p32 and Rev was also investigated...
using a protein mobility shift assay. The GST-p32-K fusion protein was labeled at the C-terminally positioned HMK site, and its ability to form complexes with Rev in a native gel electrophoresis assay was investigated (Fig. 1B). In the presence of a 2-fold molar excess of Rev to p32 fusion protein, approximately half of the p32 was shifted to a major slower migrating band (Fig. 1B, lane 4). The absence of any major higher order complexes at maximum Rev concentration (Fig. 1B, lane 7) suggests that Rev has only one major binding site on p32 and that Rev oligomerization is limited or not detectable in this assay. No differences in the Rev binding potential were observed when using GST-p32 or p32-K in the binding reaction, implying that the GST and HMK tags did not interfere with Rev interaction (data not shown). The binding of GST-p32-K protein to a synthetic peptide covering only the basic domain of Rev (Rev-(34–50)) was also investigated (Fig. 1C). Addition of a 2-fold molar concentration of Rev-(34–50) to p32 fusion protein resulted in a slower migrating complex (Fig. 1C, lane 3). At larger concentrations of Rev-(34–50) a second complex appeared, which may represent binding of multiple Rev-(34–50)s to a single p32 fusion molecule (Fig. 1C, lane 5). The relatively large shift, caused by the binding of a 17-amino acid peptide to a 518-amino acid large p32 fusion protein, may be explained by the high positive charge of the peptide. The binding of Rev-(34–50) to p32 fusion protein was also investigated in a competition assay in the presence of intact Rev protein (Fig. 1C, lanes 6–11). Rev-(34–50) efficiently competed with Rev for p32 binding, and comparable levels of p32-Rev-(34–50) and p32-Rev complexes were formed in the presence of similar molar concentrations of Rev-(34–50) to Rev (Fig. 1C, lanes 9 and 10). This result implies that Rev-(34–50) and intact Rev protein bind with comparable affinities to p32.

**Fig. 1. In vitro interaction between p32 and Rev.** A, a Coomassie-stained protein gel showing the ability of Rev to co-precipitate with immobilized GST-p32 protein. Two μg of GST-p32 and 0.45 μg of Rev protein (1:1 molar concentration) were mixed with 2 μg of bovine serum albumin and used as input for affinity selection on glutathione-Sepharose beads (lane 5). The proteins retained on the beads after binding and washings in buffers containing the denoted concentrations of NaCl (lanes 8–12) or nonionic detergent Tween 20 (lanes 13–17) were analyzed. Leaving out the GST-p32 protein (lane 6) or replacing it by GST (lane 7) essentially eliminated Rev binding. GST, GST-p32, and Rev protein were loaded in lanes 2, 3, and 4, respectively, as markers. Sizes of marker proteins (lane 1) are indicated. B, autoradiogram of a native polyacrylamide gel showing the protein band shift analysis of p32-Rev complexes. One hundred ng of radioactively labeled GST-p32-K protein (corresponding to a concentration of 0.2 μM) was incubated with the indicated concentrations of Rev protein and analyzed by native gel electrophoresis. The major discrete bands, corresponding to free GST-p32-K and GST-p32-K-Rev complex, are indicated. C, protein band shift analysis similar to that in panel B. The complexes formed between GST-p32-K (concentration of 0.2 μM) and the indicated concentration of Rev-(34–50) were analyzed in the absence (lanes 2–5) or presence of Rev (lanes 7–11). Lanes 1 and 6 are control lanes showing GST-p32-K alone. The asterisks in panels B and C indicate a complex that appeared in variable amounts depending on the p32 preparation. We suspect that it originates from the binding of Rev to a truncated p32 lacking the GST part, which is a common degradation product from GST-p32 (T. Ø. Tange, T. H. Jensen, and J. Kjems, unpublished observation).
Binding of p32 to Rev in the Presence of RRE RNA—The basic domain of Rev is likely to be involved both in the binding of the RRE RNA and the p32 protein (16, 23). To study the binding of p32 to Rev in the context of the RRE, we investigated the ability of glutathione beads to retain radioactively labeled RRE RNA in the presence of increasing amounts of Rev and GST-p32 proteins (lanes 6–11) or GST as a control (lanes 3–5). GST-p32 protein, at the indicated concentration, was incubated with increasing concentrations of Rev protein and a fixed amount of radioactively labeled RRE RNA. The total input of RRE probe in each lane is shown in lane 1.

Two ng of radioactively labeled RRE RNA was complexed with Rev, at the indicated concentrations and challenged with increasing concentrations of GST-p32 protein or GST protein as a control, according to the scheme above. The position of the free RRE probe is indicated.

Fig. 2. Investigating the p32-Rev interaction in the context of RRE. A, autoradiogram of a denaturing polyacrylamide gel showing the amount of radioactively labeled RRE RNA that was retained on glutathione-Sepharose beads in the presence of increasing amounts of Rev and GST-p32 proteins (lanes 6–11) or GST as a control (lanes 3–5). GST-p32 protein, at the indicated concentration, was incubated with increasing concentrations of Rev protein and a fixed amount of radioactively labeled RRE RNA. The total input of RRE probe in each lane is shown in lane 1. B, experiment similar to that in panel A except that Rev was replaced with Rev-(34–50) at different concentrations. C, RNA band shift analysis of the effect of GST-p32 on Rev-RRE complex formation. Two ng of radioactively labeled RRE RNA was complexed with Rev, at the indicated concentrations and challenged with increasing concentrations of GST-p32 protein or GST protein as a control, according to the scheme above. The position of the free RRE probe is indicated.

Fig. 3. Footprinting the binding site of Rev protein on p32. A, autoradiogram of a protein gel showing the protein footprint of Rev on p32. One hundred ng of C-terminally labeled p32-K protein was digested with Glu-C proteinase at two different concentrations in the presence of 1 μg of Rev protein (approximately 18 times molar excess) or the same amount of bovine serum albumin, indicated by + and −, respectively. C denotes the control lane in which Glu-C proteinase was omitted. Glu-C cleavages, which were specifically inhibited or enhanced by the Rev protein, are marked with solid and open arrows, respectively. The indicated Glu-C-specific cleavage sites were putatively identified on the basis of their relative mobility of products from other specific proteinases and from interpolations on a graph indicating the relationship between the logarithm of the mass of radioactive C-terminal fragment produced by proteolytic cleavage and migration of the corresponding band on the gel (data not shown). B, amino acid sequence of pro-p32. Glu-C-specific cleavages, which are specifically inhibited or enhanced by the Rev protein, are marked with solid and open arrows, respectively. Dotted lines indicate that the exact identification of glutamic acids in this region was uncertain, and underlining marks a region that is relatively inaccessible to all tested proteinases. Glutamic acids are denoted by boldface E, and amino acid numbering is done according to the pro-p32. The N termini of pro-p32 and the processed form of p32 are indicated by horizontal arrows.

These experiments imply that Rev is capable of bridging the RRE or IIB RNA to the p32 protein. This may either be accomplished by a single Rev molecule, interacting with the RRE and the p32 protein simultaneously or, more likely, by multiple Rev molecules bridging the two ligands by Rev oligomerization. Rev also co-precipitated with GST-p32 in the presence of Rev-(34–50), albeit with lower efficiency than observed for intact Rev (Fig. 2B). This suggests that the Rev peptide, when present at elevated concentrations, is also able to bridge the RRE RNA specifically to the p32 protein.
Formation of the GST-p32-Rev-RRE complex was also analyzed by a gel mobility shift assay (Fig. 2C). Rev forms multiple complexes with RRE in this type of assay (Fig. 2C, lanes 5 and 10) (29). The GST-p32 protein, by itself, exhibited no measurable affinity for the RRE (Fig. 2C, lane 1). The addition of increasing amounts of GST-p32 protein together with a constant amount of Rev protein gradually inhibited formation of the larger Rev-RRE complexes (Fig. 2C, lanes 6–8 and 11–13). This effect was specific to GST-p32 and was not observed with GST alone (Fig. 2C, lanes 9 and 14) or with human TATA-box binding protein (data not shown). Changing the order of addition of p32 and RNA to Rev protein or replacing the RRE probe with a single high affinity Rev binding site IIb (Fig. 3A), gave a similar result (data not shown). Surprisingly, we were not able to observe any novel complexes that could represent the ternary complexes among GST-p32, Rev, and RRE or IIb, predicted from the co-precipitation experiment (Fig. 2A). This suggests that such complexes are unstable under gel electrophoresis conditions.

Mapping of the Rev Binding Site on p32 by Protein Footprinting—The results from the protein band shift assay of Rev-p32 complexes suggested that Rev only has one major binding site on p32. To analyze this binding site in more detail, we used a protein footprinting assay, which we have previously applied successfully to map the RRE binding site on the Rev protein (30). In this approach, radioactively end-labeled protein is cleaved partially by a set of proteinases, which attack the surface of a protein. Adding a ligand to the reaction will sterically hinder the protease cleavages at the site of interaction. In addition, conformational changes, induced by the binding, may yield additional effects including enhanced cleavages. To map the protection of Rev on p32, C-terminally labeled p32-K protein, lacking the GST-fusion part, was probed with the glutamic acid-specific proteinase, Glu-C, in the absence and presence of Rev protein (Fig. 3A). In the reaction without Rev, a similar amount of bovine serum albumin protein was added as a control. The addition of bovine serum albumin by itself had no effect on the protease cleavage pattern. Since only the C-terminal fragments were visualized on the autoradiogram of the protein gel, each cleavage site could generally be assigned to a specific amino acid position in p32 on the basis of its apparent mobility in the SDS gel. Two major bands and one minor band, assigned as Glu198/Glu200, Glu208, and Glu196, respectively, were partially protected by Rev (Fig. 3, A and B). In addition, several Glu-C cleavages were specifically enhanced upon Rev binding. In particular, the two glutamic acids, Glu216 and Glu218, appearing as a double band just below the protected sites, were strongly enhanced, suggesting that this region undergoes a conformational change upon Rev binding. Interestingly, enhancements were also observed at two (at the primary sequence level) distantly positioned regions, which probably correspond to regions encompassing Glu292/Glu293 and Glu159/Glu157 (Fig. 3, A and B). These two regions flank a portion of p32 encompassing amino acids 98–152, which is relatively inaccessible to all tested proteinases, suggesting that it may form a core domain. p32 was also cleaved with Lys-C and Arg-C in the absence and presence of Rev, but no specific protections were observed using these proteinases (data not shown).

p32 Modulates the Effect of Rev on Splicing in Vitro—It has previously been demonstrated that Rev and Rev-(34–50) specifically inhibit splicing of an RRE containing mRNA (21). Since the p32 protein forms a complex with the essential splicing factor ASF/SF2, it is possible that the inhibitory effect of Rev on splicing is mediated by an interaction between Rev and p32. To approach this question, we investigated the effect of GST-p32 protein on the ability of Rev-(34–50) to inhibit splicing of an RRE containing mRNA in vitro (Fig. 4). In the absence of GST-p32, Rev-(34–50) specifically inhibited splicing of a pre-mRNA transcript containing the RRE (PIP7.A/B1/RRE) by
5-fold as compared with an internal control transcript lacking the RRE (PIP7.A) (Fig. 4, panel A, lanes 2 and 3, and panel B). Adding GST-p32 to the splicing reaction specifically restored splicing of RRE containing pre-mRNA in the presence of Rev-(34–50), without affecting the splicing efficiency of the construct lacking an RRE (Fig. 4A, lanes 4–7). At equimolar amounts of GST-p32 and Rev-(34–50), the specific inhibition of splicing dropped to about 2-fold, and at 2 times molar excess of GST-p32, splicing of the RRE containing mRNA was almost independent of Rev-(34–50) (Fig. 4B). The effect was specific to the GST-p32 protein, since adding GST protein alone had no effect (Fig. 4A, lanes 8 and 9).

**DISCUSSION**

Rev plays a major mechanistic role in switching the viral expression pattern from multiply spliced viral mRNAs to singly spliced and unspliced mRNA in the cytoplasm. In addition to the viral RNA target, the RRE, Rev interacts with cellular components in order for the mRNA to escape from the default splicing and transport pathways utilized by other mRNAs. It has recently been shown that a number of nucleoporin-like proteins interact specifically with a leucine-rich motif in Rev, constituting the nuclear export domain, and that this interaction is crucial for efficient transport of the mRNAs to the cytoplasm (9–13). In this report, we characterize a very stable in vitro interaction among another functionally important region of Rev, the basic domain, and the human ASF/SF2-associated p32 protein. p32 is an acidic protein, rich in glutamic and aspartic acid residues, which raises the possibility that p32 interacts with the highly basic region of Rev protein through unspecific ionic interactions. Although this possibility is difficult to disprove, particularly since simple ionic interactions often play an important role in molecular recognition, several of our observations suggest that the molecular recognition is specific and biologically relevant: (a) the p32-Rev complex was resistant to salt concentrations up to 750 mM, which destabilizes ionic interactions, (b) the p32-Rev complex appeared as a single major band on a native gel over a large titration range, and (c) only one short stretch of glutamic acids, out of several highly acidic regions in p32, was specifically protected by Rev. Furthermore, in vivo studies have shown that transient expression of the murine homologue of p32, YL2, potentiated the function of Rev up to 4-fold and that antisense YL2 transcripts abolished Rev function (23, 33).

Since both p32 and the RRE interact with the basic domain of Rev, one may suspect that simultaneous binding of p32 and RRE to the Rev protein is impaired. The observation that Rev was capable of bridging p32 to the RRE in a solution binding assay, therefore suggests that Rev forms oligomers in such a manner that distinct basic regions can interact with the RRE and p32 independently. However, in the RNA mobility shift assay we found that p32 competed with Rev for binding, and we were unable to detect any ternary complex implying a simultaneous association of p32, Rev, and RRE. We suspect that a reason for this discrepancy is an instability of the Rev-Rev interactions under the applied electrophoresis conditions. In favor of this interpretation is the previously reported differences between solution binding and gel shift assays. Whereas Rev tends to form RNA independent oligomers in solution to a variable extent, when analyzed by gel filtration or chemical cross-linking (18, 19, 32, 33) only a single complex is seen between Rev and a high affinity binding site (IIB RNA), using native gel electrophoresis (16, 34–36).

A number of other proteins have been found to interact with p32. Originally, p32 was characterized as being a component of the ASF/SF2 splicing activity purified from HeLa cells (24). Subsequently, it was shown that p32 was dispensable for the general splicing activity, although the possibility cannot be ruled out that p32 has a more specialized role in splicing (37). Recent evidence suggests that p32 also interacts with the HIV-1 Tat protein (38–40). In one report, a Tat-binding protein (TAP) was isolated on the basis of Tat affinity chromatography, and the sequence turned out to be identical to p32 except for a few amino acids in the N-terminal precursor segment (39). The same group also found that TAP (p32) interacts with the C terminus of TFIIIB, and it was suggested that TAP (p32) may function as a cellular co-activator that bridges Tat to the general transcription machinery (38). The significance of the TAP (p32)-Tat interaction was substantiated by a two-hybrid analysis, in vitro binding studies, and a demonstration implying that TAP (p32) was able to cooperate with Tat to synergistically stimulate transcription (38, 39). The region involved in Tat binding was mapped to amino acids corresponding to 247–282 in p32, which is outside the region where we see protection by Rev (amino acids 196–208). Also, it was found that TAP (p32) primarily interacted with a 17-amino acid conserved core segment of the Tat activation domain, whereas the basic domain of Tat was dispensable and by itself unable to bind (38). Together, these data suggest that Tat and Rev may interact with p32 in different ways and raises the possibility that p32 plays multiple roles in HIV-1 replication.

Several lines of evidence suggest that the basic domain of Rev plays a direct functional role other than RNA binding, nuclear localization, and protein oligomerization. First, it has been demonstrated that, even when multiple Rev molecules were tethered to the mRNA through heterogeneous RNA binding sites, the basic domain was not dispensable for Rev function (41, 42). Secondly, the basic domain alone can specifically inhibit splicing of RRE containing transcripts in vitro, suggesting a role in RNA splicing (21). The binding potential of the basic domain of Rev for p32 and the RRE suggests that p32 is a cellular co-factor for Rev. The association of the p32 protein with ASF/SF2, which binds in a cooperative fashion with U1 snRNP to the 5'-splice site, theoretically places Rev and p32 on the same mRNA. It is therefore likely that Rev, sequestered on the RRE, is able to interact with p32 and directly influence the splicing process (Fig. 5). In this report, we show that equimolar amounts of GST-p32 and Rev-(34–50) diminished the inhibitory effect of Rev-(34–50) on splicing. A possible explanation for this antagonizing effect of p32 in this assay may be that exogenously added p32 squelches the functional interaction between Rev peptide and endogenous p32, associated with ASF/SF2, and thereby inhibits Rev function. An alternative explanation, which cannot be excluded, is that p32 may disrupt the binding of Rev-(34–50) to the RRE, thereby relieving the inhibition of splicing.

A prediction from the model shown in Fig. 5 would be that Rev may increase the stability of the U1 snRNP interaction
with the 5'-splice site and thereby inhibit the subsequent displacement of U1 snRNP, which is necessary for U4/U6/U5 tri-snRNP to enter the spliceosome (43). In support of this model, it has previously been shown that Rev specifically increases the amount of U1 snRNP in prespliceosomes formed on RRE containing mRNAs and efficiently blocks the entry of the U4/U6.U5 tri-snRNP (22). Furthermore, in vivo experiments have demonstrated that Rev regulation of a construct, containing an intron, requires that the 5'-splice site be recognized by U1 snRNP and probably also ASF/SF2 (44–46).

In conclusion, Rev most likely functions both at the level of splicing and at the level of transport, through the basic domain and the nuclear export signal, respectively. Further investigations of the functional roles of the interaction between p32 and the nuclear export signal will be crucial for understanding Rev function and provide a valuable tool to study the functional interplay between splicing and transport in general.

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