Mutations in the Nuclear Export Signal of Human Ran-binding Protein RanBP1 Block the Rev-mediated Posttranscriptional Regulation of Human Immunodeficiency Virus Type 1*

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We identified a region in the human Ran GTPase-binding protein RanBP1 that shares similarities to the cAMP-dependent protein kinase. Mutational analysis confirmed that this region is responsible for the cytoplasmic accumulation of RanBP1 and can functionally replace the nuclear export signal of Rev of human immunodeficiency virus type 1. We showed that RevBP1 interferes with Rev-mediated expression of human immunodeficiency virus type 1, whereas the RanBP1 with inactivated nuclear export signal abrogates Rev function. Expression of a Rev-independent molecular clone, which is regulated via the constitutive transport element (CTE) of the simian retrovirus type 1, is not affected. These findings indicate that Rev and RanBP1 compete for the same nuclear export pathway, whereas Rev and the CTE-mediated pathways are distinct. The inhibition of Rev function is independent of the ability of RanBP1 to associate with Ran and therefore, it is not likely a result of interference with Ran function. These data suggest that RanBP1 interacts with Rev at the putative nuclear receptor and, hence, shares a step in posttranscriptional pathway with Rev.

One of the best studied examples of regulation at the posttranscriptional level is that of the Rev-responsive element (RRE)1-containing mRNAs of HIV-1 (for reviews, see Refs. 1–5). The Rev protein binds to the RRE and promotes the export of the RRE-containing mRNA to the cytoplasm and their expression. Rev contains an RNA-binding domain and a nuclear export signal (NES). Mutations in the NES are transdominant (TD) and lead to inhibition of Rev’s nucleocytoplasmic export resulting in inhibition of HIV expression. Nucleoporin-like proteins hRip/Rab have been shown to interact with the NES of Rev and are believed to be involved in the nuclear export of Rev (6, 7). Similar NES elements have been identified in the inhibitor of cAMP-dependent protein kinase (PKI) (8) and the 5 S RNA-binding protein TFIH1A (9) and have been shown to functionally replace the NES of HIV-1 Rev. We identified a related NES region in the Ran GTPase-binding protein 1 of human (hRanBP1) (10, 11) and mouse RanBP1/HTF9-A (mRanBP1) (12) origin. They belong to a family of proteins that bind to the GTP-bound Ran that includes known and putative nucleoporins as well as proteins of unknown function (13–19). Ran GTPase is required for active transport of macromolecules through the nuclear pore (for recent reviews, see Refs. 20 and 21). Here, we show that the NES signals of RanBP1 and Rev functionally cross-interfere, indicating a competition for a common export pathway. NES(–)–RanBP1 mutants completely abrogate Rev-mediated regulation of HIV-1 without affecting the regulation mediated by the posttranscriptional control element (CTE) of simian retrovirus type 1. Taken together, our data suggest that RanBP1 and Rev share a nuclear export pathway, which is distinct from the CTE-mediated pathway.

EXPERIMENTAL PROCEDURES

Recombinant DNA and Transfections—HIV-1 molecular clone pNL4-3 (22), the rev(−) clone pNL4-3B (23, 24), the Rev-independent pNL43 Rev(−)R(−)S containing the CTE of SRV-1 (25), the rev expression plasmid pBrev (26), the TD Rev expression vectors pRevBL (27) and pRevM10BL (28) have been described. To generate hybrid Rev-NES proteins, the Psi-BoEII fragment of pBrev was replaced by synthetic DNA encoding the heterologous NES as shown on Fig. 1, substituting amino acids 72–88 of Rev. The RanBP1 gene was cloned by polymerase chain reaction (PCR) using cDNA from human Jurkat cells. Mutations in the amino acids and RBD domain of RanBP1 were introduced by PCR. The RanBP1 expression plasmids were constructed by insertion of the PCR fragments into the BssHII-RanH1 sites of pB7T7 (29). The GFP-containing plasmids have an insertion of the mutant sg25GFP gene3 into the BssHII site generating the N-terminal GFP-RanBP1 fusion proteins consisting of GFP followed by a linker (Ala-Pro-Ala) and RanBP1. All recombinant genes were sequenced on both strands. HLTat is a HeLa-derived cell line that constitutively produces Tat protein (31). 293 is a human embryonic kidney cell line. Cells were transfected by the calcium phosphate coprecipitation technique, and total protein was extracted as described (25). pRSVLuc (32) or pLuc1uc (33) luciferase expression plasmids were included in the transfection mixtures, and the luciferase activity was measured as described (33). GFP fluorescence was measured in cell lysates using CytoFluor fluorimeter (28).

Western Blots and Immunoprecipitations—Western blots were performed with ECL kit (Amersham Corp.), using rabbit anti-GFP sera2 and anti-Ran monoclonal antibody (Transduction Laboratories). For native immunoprecipitation, HLTat cells transfected with GFP-RanBP1 expression plasmids were lysed in 10 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 2.5 mg/ml heparan by two freeze-thaw cycles, treated with 10 units/ml DNase I for 15 min at room temperature, cleared by centrifugation at 13,000 rpm for 10 min at 4 °C and gel-filtrased on Sephadex G50 against 0.9% Tween 20 in PBS (PBS-T). Rabbit anti-GFP or normal sera were captured to protein A-Sepharose in PBS for 1 h at 4 °C; the beads were then blocked in 10% milk, 1% casein, 1% gelatin, and 0.2% Tween 20 in PBS for 1 h at 4 °C. The beads were incubated with the cell extracts for 3–4 h at 4 °C, washed with PBS-T containing 1 mM EDTA,
FIG. 1. Comparison of the nuclear export signals of RanBP1, PKI, and Rev. To search for the regions homologous to the NES of PKI, the published sequences of mouse and rat PKI isoforms α and β were aligned, and the region of the alignment spanning the NES was used to create a profile to search a protein sequence data base. Multiple sequence alignments, profile construction, and data base searches were performed with the PILEUP, PROFILEMAKE, and PROFILESERACH programs of the University of Wisconsin Genetic Computer Group (UWGCG) package. The NESs of human and mouse RanBP1 and the rabbit and mouse PKI proteins are compared, and the derived consensus is given. The consensus NES of Rev (30) is shown. NES(–) mutant contains alanine substitutions (underlined) replacing conserved hydrophobic residues (shown in bold) of human RanBP1.

FIG. 2. NES of hRanBP1 and mRanBP1 can functionally substitute for that of Rev. Human 293 cells were transiently cotransfected with 2 μg of pNL4-3fB and 0.3 μg of the indicated hybrid Rev expression plasmids. All transfection mixtures contained 0.1 μg of luciferase expression plasmid RSVluc. After 24 h, the cells were harvested and Gag production (black bars) and luciferase activity (gray bars) were measured. Expression in the presence of Rev was normalized to 100%. Mean numbers and S.E. for triplicate plates for a representative experiment are shown. Similar data were obtained in four independent transfection experiments.

RESULTS AND DISCUSSION

By data base searches for similarity to the nuclear export signals of different PKI proteins, we identified a region in the human and mouse Ran GTPase-binding protein 1 (RanBP1) lying outside the RBD. Comparison of these regions with other NES, including that of Rev of HIV-1 and the consensus NES of Rev, revealed conservation of a functionally important pattern of hydrophobic residues (Fig. 1). To study the function of the identified NES-like sequence, we generated hybrid proteins that have the Rev export signal replaced with the NES-like elements of the human and mouse RanBP1 and, as control, with the previously described NES of PKI. We then tested whether these proteins can activate gag expression from a rev molecular clone of HIV-1 (Fig. 2). In the absence of Rev or in the presence of a transdominant Rev (RevBL) Gag expression was barely detectable, whereas in the presence of Rev high level of Gag was produced. Comparison to wild type Rev showed that the Rev hybrid proteins containing the NES of human or mouse RanBP1 had about 10% activity, whereas Rev containing the PKI export signal showed 20% activity. Indirect immunofluorescence analysis further revealed that the Rev-RanBP1 hybrid protein localized in the nucleoli and translocated to the cytoplasm upon actinomycin D treatment like Rev (not shown). Hence, the identified element from RanBP1 can efficiently replace the activation/nuclear export signal of Rev, and the resulting hybrid protein has the characteristic properties of Rev.

We next studied the role of the NES for the localization of the human RanBP1. We generated a RanBP1 fusion with GFP and also introduced mutations within the NES region (L183A, L186A, and V188A; see Fig. 1), generating NES(–)RanBP1. The GFP-tagged wild type and mutant proteins were studied upon transient transfection in living cells (Fig. 3). In HLat cells, a HeLa-derived cell line, the wild type GFP-hRanBP1 localizes to the cytoplasm and is excluded from the nucleus. Confocal microscopy and immunogold electron microscopy confirmed that the wild type protein is not detectable in the nucleus (not shown). In contrast, the NES(–) mutant protein was found mostly in the nucleus as well as in the cytoplasm. Thus, the NES(–) mutation inactivates the nuclear export signal. Similarly, in human 293 cells, the wild type GFP-hRanBP1 was found in the cytoplasm. Although the localization of the NES(–)RanBP1 was shifted toward the nucleus, we also detected many cells where the protein is predominantly cytoplasmic. The difference in the effect of NES mutation in these two cell types is unclear. After completion of our study, Richards et al. (34) published similar data showing that cytoplasmic localization of RanBP1 is determined by a NES. In their study, L186A or V188A mutations resulted in nuclear accumulation of RanBP1 in hamster BHK21 cells (34). Taken together, these
data suggest that RanBP1 is a nucleocytoplasmic shuttle protein.

To explore whether the localization of RanBP1 depends on its interaction with Ran, we introduced mutations (K93A, R95A, G96A, and G98A) in the RBD of the GFP-tagged RanBP1 and NES(-)RanBP1. These changes affect amino acids that are well conserved between different Ran-GTP-binding proteins (14). Coimmunoprecipitation assays under nondenaturing conditions (Fig. 4) were used to confirm the inability of the RBD mutants to associate with Ran. Western immunoblots verified similar levels of Ran (Fig. 4A) and GFP-RanBP hybrid proteins (Fig. 4B) in the different lysates. While GFP-RanBP1 has an expected apparent molecular mass of about 50 kDa, mutation of NES affected the electrophoretic mobility of the protein. We also detected faster migrating proteins produced from all expression vectors, that may represent N-terminal truncations within GFP moiety (Fig. 4B). The same extracts were immunoprecipitated with either preimmune serum (Fig. 4C) or anti-GFP antiserum (Fig. 4D) and analyzed on Western immunoblots using the anti-Ran antibody, which confirmed that only wild type RanBP1 and the NES(-) mutant protein associate with Ran, whereas the mutation of RBD abolished this interaction. Subcellular localization studies (Fig. 3) in HLtat and in 293 cells showed that RBD(-)RanBP1 localizes primarily to the cytoplasm, like the wild type protein, demonstrating that this localization is not dependent on the interaction with Ran. The RBD(-) mutation also did not affect the nuclear accumulation of the NES(-)RanBP1 in HLtat cells. In 293 cell line, the

FIG. 5. The cytoplasmic accumulation of RanBP1 can be inhibited by Rev but not by trans-dominant Rev. HLtat cells were transiently transfected with 2 µg of plasmids expressing GFP-hRanBP1 (A–D) or GFP-NES(-)hRanBP1 (E and F). The cells were cotransfected with 5 µg of the rev expression plasmid pBSRev (A–C and E) or the trans-dominant rev expression plasmid pBSRevM10BL (D and F). Fluorescent microscopy was performed 24 h posttransfection. Similar data were obtained in four independent experiments.

FIG. 6. RanBP and NES(-)RanBP1 specifically inhibit the Rev-mediated posttranscriptional regulation. Human 293 cells were transfected and analyzed for Gag production and luciferase activity as in Fig. 2. The transfection mixtures included 1 µg of pNL4-3 B supplemented with 0.05 µg of pBSRev (Rev-dependent) or 1 µg of NL43 Rev(-)R(-)S (Rev-independent) and increasing amounts of GFP-RanBP1 or GFP-NES(-)RanBP1 expression vectors (plotted on x axis as micrograms in A–E). All mixtures also contained 0.1 µg of the luciferase expression plasmid pRSVLuc. Gag (solid lines) and luciferase (dashed lines) values in the absence of RanBP1 plasmids is 100%. E, the expression of GFP fusion proteins was quantitated in the lysates of transfected cells with CytoFluor fluorimeter (raw CytoFluor units shown on the y axis). Similar data were obtained in five independent transfection experiments.
RBD(−) mutation led to a predominant nuclear accumulation of NES(−)RanBP1 in all cells. These data indicate that the RBD and NES determinants of RanBP1 may have different strength in different cell types. Taken together, these experiments suggest that the ability to bind Ran does not contribute significantly to the shuttling properties of RanBP1.

The similarity of the nuclear export signals of RanBP1 and Rev suggests that these proteins rely on a common nuclear export pathway. To test this possibility, we studied the effect of Rev on the subcellular localization of GFP-hRanBP1 in living cells (Fig. 5). We found hRanBP1 to shift from the cytoplasmic to the nuclear localization in the presence of Rev (Fig. 5, A−C). As a result, fluorescence was found over the entire cell (Fig. 5A, a typical cell is indicated by arrow) or was predominantly nuclear (Fig. 5C). In contrast, the presence of TD Rev protein M10BL had no effect on RanBP1 localization (Fig. 5D).

Immunoblot analyses performed on extracts prepared from the same cells showed comparable levels of Rev and RevM10BL proteins (not shown), which excludes the possibility that the relative amount of Rev or RevM10BL is responsible for this finding. In parallel experiments, we showed that the localization of the NES(−)RanBP1 was not affected by Rev or RevM10BL (Fig. 5, E and F). Taken together, these data demonstrated that Rev interferes with the nuclear export of hRanBP1 via its nuclear export signal. Therefore, these findings suggest that Rev and RanBP1 compete for a common NES-specific export pathway.

Given the finding that Rev interferes with the nuclear export of RanBP1, we explored whether RanBP1 and its mutants affect Rev function as measured by inhibition of Gag production of HIV-1. We cotransfected the rev− molecular clone of HIV-1 in the presence of Rev expression vector and increasing amounts the wild type and NES(−)RanBP1 expression plasmids (Fig. 6). The presence of both wild type RanBP1 and NES(−)RanBP1 led to greatly reduced levels of Gag production. While the presence of excess RanBP1 lowered HIV expression about 10-fold (Fig. 6A), the presence of NES(−)RanBP1 resulted in a dose-dependent complete inhibition (Fig. 6B) of Gag expression. Similar data were obtained using the intact HIV-1 molecular clone NL43 (data not shown). The specificity of this inhibition was controlled for by cotransfection of a luciferase expression vector, driven by the HIV-1 or Rous sarcoma virus long terminal repeat promoter, which showed no inhibition in the presence of either wild type or mutant RanBP1 (Figs. 6, A and B, dotted lines). To control for the levels of wild type and NES(−)RanBP1 expression, we measured GFP fluorescence in the lysates (Fig. 6E) and demonstrated that similar levels of these proteins were expressed. Therefore, the distinct effects observed for the wild type and the NES(−)RanBP1 reflect intrinsic properties of the two proteins. In summary, our results indicate that RanBP1 interferes with the posttranscriptional regulation by Rev, and vice versa, Rev, via its own nuclear export signal, interferes with the nuclear export of RanBP1. Therefore, the two proteins are likely to compete for NES-specific components of the same export pathway. The strong inhibitory effect of NES(−)RanBP1 on Rev function has been an unexpected result, since such mutants are not predicted to associate with and, hence, to compete for the putative NES receptors. This led us to propose that NES(−)RanBP1 directly targets the NES-specific pathway through interaction with the endogenous RanBP1, directly or through interaction with another common factor. The presence of NES(−)RanBP1 in such a complex may interfere with its transport, resulting in the observed inhibition of Rev activity. A similar mechanism has been described for the inhibition of Rev function by TD Rev (28, 35). Our model would predict that the inhibitory activity of wild type and NES(−)RanBP1 will specifically affect this NES-specific pathway, but not other regulated nuclear export. Therefore, we tested whether the presence of RanBP1 or its NES-mutant affect expression of the Rev-independent HIV-1 molecular clone. This clone is Rev− and RRE− and contains the CTE posttranscriptional control element of SRV-1 replacing the Rev/RRE system (25, 29). As shown in Figs. 6C and 6D, the expression of the Rev-independent HIV-1 was not affected by RanBP1 or the NES(−)RanBP1. These data further suggest that RanBP1 and Rev share a specific nuclear export pathway, which is distinct from the CTE-mediated export pathway.

We asked whether the inhibitory effect of RanBP1 depends on the ability to associate with Ran and tested the effect of the RBD(−)RanBP1 proteins on the expression of the HIV-1 (Fig. 7). Saturating amounts of plasmids expressing RBD(−)-RanBP1 or RBD(−)NES(−)-RanBP1 as well as RanBP1,NES(−)-RanBP1, or GFP alone were cotransfected with Rev-regulated (Rev-dependent; Fig. 7, bottom) or CTE-regulated (Rev-independent; Fig. 7, top) molecular clones, and the Gag production was measured with antigen capture assay. Measurements of GFP fluorescence in the lysates showed comparable levels of expression for different mutant proteins (not shown). RBD mutation had no significant effect on the inhibitory activity of wild type and NES(−)RanBP1, suggesting that the interaction of RanBP1 with Rev does not play a role in its specific effect on Rev function. The NES(−)RBD(−)RanBP1 inhibited HIV expression reproducibly slightly less than NES(−)RanBP1. However, this difference (about 6-fold) is small as compared with 400-fold inhibition obtained by NES(−)RanBP1 and may be attributed to an overall structural effect of the RBD(−) mutation rather than the loss of the interaction with Ran. Similar data were obtained from dose-dependent inhibition studies (data not shown). In summary, these data indicate that the inhibition of Rev function by RanBP1 is directly mediated through its nuclear export signal and is independent of RanBP1’s interaction with Ran.

Ran GTPase has been implicated in nuclear export, although the molecular mechanisms of such activity are not known. It is not clear how the ability of RanBP1 to shuttle is related to its proposed function as a participant of nuclear localization sig-
nal-mediated nuclear import. It should be noted that removal of the region containing NES did not affect the ability of RanBP1 to enhance the binding of Ran-GTP to β-karyopherin in vitro (36) and therefore NES is dispensable for this reported function. However, the presence of NES may suggest a role of RanBP1 in the cytoplasmic delivery of GTP-bound form of Ran.

In addition, the ability of RanBP1 to interact with both Ran-GTP and the NES-specific components of a nuclear export pathway makes it a candidate physical link between Ran and nuclear export. The likely role of RanBP1 in this model is to tether active, nuclear form of Ran to the putative export intermediates that are involved in trafficking of other NES-containing substrates, such as Rev of HIV-1.

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