hRIP, a cellular cofactor for Rev function, promotes release of HIV RNAs from the perinuclear region

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Human immunodeficiency virus Rev facilitates the cytoplasmic accumulation of viral RNAs that contain a Rev binding site. A human Rev-interacting protein (hRIP) was originally identified based on its ability to interact with the Rev nuclear export signal (NES) in yeast two-hybrid assays. To date, however, the function of hRIP and a role for hRIP in Rev-directed RNA export have remained elusive. Here we ablate hRIP activity with a dominant-negative mutant or RNA interference and analyze Rev function by RNA in situ hybridization. We find, unexpectedly, that in the absence of functional hRIP, Rev-directed RNAs mislocalize and aberrantly accumulate at the nuclear periphery, where hRIP is localized. In contrast, in the absence of Rev or the Rev cofactor CRM1, Rev-directed RNAs remain nuclear. We further show that the RNA mislocalization pattern resulting from loss of hRIP activity is highly specific to Rev function: the intracellular distribution of cellular poly(A)⁺ mRNA, nuclear proteins, and, most important, NES-containing proteins, are unaffected. Thus, hRIP is an essential cellular Rev cofactor, which acts at a previously unanticipated step in HIV-1 RNA export: movement of RNAs from the nuclear periphery to the cytoplasm.

[**Keywords:** Rev; HIV-1; RNA export; hRIP]

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Animal viruses often encode regulatory proteins, which facilitate expression of their viral genes in the host cell. One such example is the human immunodeficiency virus type 1 (HIV-1) Rev protein, which uses a novel mechanism to regulate viral messenger RNA (mRNA) export from the nucleus. Rev is a sequence-specific RNA binding protein that facilitates the cytoplasmic accumulation of the intron-containing HIV-1 *gag-pol* and *env* mRNAs (Cullen 2002). Rev interacts with a *cis*-acting viral RNA element in these mRNAs designated the Rev responsive element (RRE; Pollard and Malim 1998; Hope 1999). Rev contains two distinct functional domains: an arginine-rich RNA binding motif (ARM) and a leucine-rich “effector” domain. Rev’s ARM domain confers sequence-specific RNA binding, protein oligomerization, and nuclear targeting (Frankel and Young 1998). Rev’s effector domain contains a leucine-rich nuclear export signal (NES), which binds the nuclear export receptor CRM1 (Weis 2002). Other cellular proteins have been shown to interact directly or indirectly with the Rev NES, including the kinesin-like Rev/Rex effector binding protein (REBP) and the eukaryotic initiation factor-5

alpha (eIF-5α; Bevec et al. 1996; Dayton 1996; Kjems and Askjaer 2000; Venkatesh et al. 2003).

The human Rev-interacting protein (hRIP), also known as hRab or Hrb, was identified using a yeast two-hybrid screen with Rev as the bait (Bogerd et al. 1995; Fritz et al. 1995). Additional studies demonstrated a strong correlation between the ability of Rev NES mutants to support Rev function in yeast and their ability to interact with hRIP in the two-hybrid assay (Stutz et al. 1996). However, subsequent genetic analyses indicate the Rev–hRIP interaction is indirect, and is likely bridged by CRM1 (Fritz and Green 1996; Henderson and Percipalle 1997; Neville et al. 1997). hRIP contains multiple phenylalanine-glycine (XXFG) repeat sequences reminiscent of the GLFG and XFXFG repeats found in most yeast and mammalian nuclear pore proteins also known as nucleoporins or “Nups” (Doye and Hurt 1997; Strambio-de-Castillia et al. 1999). The FG repeats in nucleoporins are believed to mediate protein–protein interactions, and in the case of hRIP, are required for its interaction with Rev in vivo. At its N terminus, hRIP contains a zinc finger motif (Krishna et al. 2003), which shares a high degree of sequence identity with a subclass of C₄H₂-type zinc-finger domains found in proteins involved in vesicular trafficking, intracellular protein localization, cytoskeletal rearrangement, RNA metabolism, or signal transduction (Cukierman et al. 1995;

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Figure 1. Construction of hRIP derivatives. Schematic representation of N- and C-terminally truncated and fluorescent hRIP derivatives. (ZnF) C_4H_2 -type zinc finger domain; (S/TRR) serine/threonine-rich region; (FGM) phenylalanine-glycine motifs; (arrowhead) asparagine-proline-phenylalanine (NPF) motifs; (EGFP) enhanced green fluorescent protein; (WT) hRIP; (aa) amino acids.

Kirchhausen 2000; Premont et al. 2000; Turner et al. 2001; Nie et al. 2002; Dubois et al. 2003). Within the central body of the protein are stretches of serine/threonine-rich sequences (S/TRR), several of which are substrates for a subset of cellular kinases, and thus, possible targets for regulation (Maraldi et al. 1999; Parker and Parkinson 2001; Biondi and Nebreda 2003). Lastly, there are several asparagine-proline-phenylalanine (SS/TN-PFXX) sequence motifs interspersed among the FG repeats within the most terminal third of the protein. Such NPF motifs are thought to mediate interactions with distinct members of the Eps 15 homology (EH)-domain protein family, which also interact with CRM1 and other nuclear components (Santolini et al. 1999; Hyman et al. 2000; Poupon et al. 2002). Recent microscopic and biochemical experiments indicate that hRIP is localized predominantly at the nuclear periphery, perhaps in a novel perinuclear compartment (Doria et al. 1999).

Based on its putative structural domains and intracellular distribution, hRIP has properties consistent with a role in RNA trafficking or localization. To date, however, the cellular function of hRIP and a role for hRIP in Rev function have remained elusive. Here we describe complementary genetic approaches to inactivate hRIP

and analyze the effect of hRIP ablation on Rev function. We find that hRIP is, in fact, an essential cellular cofactor for HIV-1 Rev activity that acts at an unanticipated step in Rev-directed RNA export. Our results have direct relevance to Rev function and general implications for cellular RNA trafficking.

Results

A dominant-negative hRIP mutant interferes with Rev function

To analyze the function of hRIP, we devised a strategy similar to those traditionally used to study proteins of unknown function, namely, the identification and characterization of a dominant-negative form of hRIP. To accomplish this, we generated a series of N- and C-terminally truncated hRIP derivatives by the systematic removal of the putative hRIP functional domains (Fig. 1). Because hRIP has properties that are consistent with a cellular Rev cofactor, we tested the activity of these derivatives using a standard assay for Rev function (Hope et al. 1990). This mammalian cell transfection assay uses a translation-based reporter gene (pCMV128) that has a single intron containing both the HIV-1 RRE and the bacterial chloramphenicol acetyltransferase (CAT) cDNA as an indicator of Rev function. This intron is efficiently spliced, and therefore cells transfected with pCMV128 express only trace amounts of CAT enzyme activity (Fig. 2A, lane 1). Cotransfection with a Rev expression plasmid (pcRev; Malim et al. 1989) causes unspliced transcripts to enter the cytoplasm, and thus CAT expression is increased (Fig. 2A, lane 2). Most of these hRIP derivatives had no effect; we did, however, identify one, hRIP Δ N360, which appeared to interfere with Rev function in this assay (Fig. 2A, lanes 3–5). This hRIP derivative, which retains the Nup-homology region of

Figure 2. A dominant-negative hRIP mutant interferes with Rev function. (A) A transient transfection assay for Rev function. The CAT reporter plasmid pCMV128 and pCMV β -gal were cotransfected with the Rev expression plasmid pcRev or pcRev and the indicated hRIP expression plasmid into Cos-1 cells. CAT and β -galactosidase enzyme activities were assayed as described in Zapp et al. (1993). (–) Absence; (+) presence; (WT) hRIP. (B) The hRIP Δ N360 mutant inhibits Rev function in a dose-dependent manner. Cos-1 cells were cotransfected with pCMV128, pcRev, pCMV β -gal, and increasing amounts of hRIP Δ N360. Enzyme activities were assayed as in (A). (–) Absence; (+) presence.

hRIP required for interaction with Rev in vivo, inhibited Rev function in a dose-dependent manner (Fig. 2B). Because hRIP Δ N360 exerted a negative effect on Rev function in the presence of endogenous hRIP, we conclude that it acts as a *trans*-dominant inhibitor of hRIP function.

To investigate the nature of the block exerted by hRIP Δ N360 on Rev function, we analyzed the intracellular distribution of Rev-directed RNAs using in situ hybridization. Cos-1 cells were cotransfected with the subgenomic HIV-1 *tat* expression plasmid (pgTAT; Malim et al. 1989) and pcRev, in the presence of phRIP or phRIP Δ N360. To confirm the specificity of Rev function in this assay, we cotransfected cells with pgTAT and a plasmid that expresses the RevM10 *trans*-dominant inhibitor (pcRevM10; Malim et al. 1989). Alternatively, cells were cotransfected with pgTAT and pCMV Δ CAN, a plasmid that expresses a truncated form of the nucleoporin CAN/Nup214 (Δ CAN). Δ CAN blocks the ability of CRM1 to interact with the nuclear pore complex (NPC) and inhibits the nuclear export of Rev (Fornierod et al. 1997; Bogerd et al. 1998; Yi et al. 2002). The intracellular

distribution of *tat* RNA was visualized using a fluorochrome-labeled oligonucleotide probe complementary to stem loop IIB of the HIV-1 RRE. In the absence of Rev, *tat* RNAs were nuclear localized (Fig. 3A, first row, second panel). Rev promoted the cytoplasmic accumulation of *tat* mRNAs in an NES- and CRM1-dependent manner (Fig. 3A, first row, third and fourth panels; bottom row, second panel). Cells transfected with reagent alone or an empty DNA vector (pCMV) contained no fluorescent signals after hybridization with the probe, confirming the detection was specific for *tat* RNAs (Fig. 3A, first and second rows, first panel). Overexpression of hRIP had no discernible effect on the cytoplasmic accumulation of *tat* RNAs (bottom row, third panel). In contrast, the *tat* mRNAs were mislocalized and aberrantly accumulated at the nuclear periphery in the presence of hRIP Δ N360 (Fig. 3A, second row, fourth panel).

Using the same approach, we tested whether hRIP Δ N360 could interfere with the cytoplasmic accumulation of any Rev-directed RNA. We inserted a high-affinity Rev binding site into the U6 small nuclear ribo-

Figure 3. The hRIP Δ N360 mutant causes Rev-directed RNAs to mislocalize and aberrantly accumulate at the nuclear periphery. (A) The subgenomic HIV-1 *tat* expression plasmid (pgTAT) was cotransfected with pcRev or pcRev and the indicated hRIP expression plasmid into Cos-1 cells. Control transfections contained pgTAT alone, pgTAT and pCMV, pgTAT and pRevM10, or pgTAT and p Δ CAN. The intracellular distribution of *tat* RNA was analyzed by in situ hybridization using a Cy3-labeled RRE-specific probe. (B) The U6-RRE expression plasmid (pU6RRE) was cotransfected with pcRev, or pcRev and phRIP Δ N360 into Cos-1 cells. Control transfections contained pCMV alone or pU6RRE and pCMV. The intracellular distribution of U6RRE RNA was analyzed as described in A.

nucleoprotein RNA (*U6snRNA*) to generate a sequence-minimized RNA polymerase III (pol III)-derived transcript (*U6RRE*), which is exported from the nucleus in a Rev-directed manner. When expressed from its cognate promoter, the *U6RRE* transcript is present at high levels in the nuclei of mammalian cells (Fig. 2B, second panel). Cos-1 cells were cotransfected with a *U6RRE* RNA expression plasmid (p*U6RRE*) and pcRev in the absence or presence of *hRIPΔN360*, and the intracellular distribution of the *U6RRE* RNA analyzed as in the previous experiment. Figure 2B shows that Rev efficiently promoted the cytoplasmic accumulation of *U6RRE* transcripts in the absence of *hRIPΔN360* (third panel). In the presence of *hRIPΔN360*, however, these RNAs were mislocalized and aberrantly accumulated at the nuclear periphery. Thus, the intracellular distribution of *U6RRE* RNAs was strikingly similar to that of *tat* mRNAs in the presence of *hRIPΔN360*.

Next, we used differential interference contrast (DIC) microscopy to define the perinuclear localization of Rev-directed RNAs more precisely. Cos-1 cells were cotransfected with pgTAT, pcRev, and *hRIPΔN360* and in situ RNA hybridization analysis was performed as in the previous experiments. The results in Figure 4 clearly show that the accumulation of Rev-directed RNAs is on the outside of the nucleus (Fig. 4, third and fourth rows). Collectively, our results indicate that *hRIPΔN360* exerts its inhibitory activity on RRE-containing RNAs at the cytoplasmic side of the NPC. This previously unanticipated step further implies an extended role for Rev in the movement of Rev-directed RNAs from the nuclear periphery to the cytoplasm.

We next performed a series of experiments to confirm that the inhibition exerted by *hRIPΔN360* on Rev-directed RNA export was specific. The presence of both a nuclear localization signal (NLS) and an NES enables Rev to shuttle continuously between the nucleus and cytoplasm, an activity required for its function (Kalland et al. 1994; Meyer and Malim 1994; Richard et al. 1994). In view of this requirement, we examined whether *hRIPΔN360* could interfere with the general NLS-dependent protein import or NES-dependent protein export pathways. Cos-1 cells were cotransfected with *hRIPΔN360* and an HIV-NLS- or HIV-NES-containing enhanced green fluorescent protein (EGFP) expression plasmid (pHIV-NLS-EGFP-NLS, pHIV-NES-EGFP; Dupont et al. 1999). The intracellular distributions of these HIV-EGFP fusion proteins and *hRIPΔN360* were analyzed by fluorescence microscopy. Consistent with previous findings, HIV-NLS-EGFP was localized in the nucleus, and HIV-NES-EGFP in the cytoplasm of transfected cells (Fig. 5A). Importantly, the intracellular distributions of these compartment-restricted reporter proteins were unaffected by *hRIPΔN360*, which was localized at the nuclear periphery (Fig. 5A, first and second rows, center and right panels). Similarly, *hRIPΔN360* had no effect on the intracellular distribution of ectopically expressed Rev, endogenous CRM1, and other NES-containing cellular proteins in subsequent localization studies (data not shown). These results provide evidence

pgTAT + pcREV + *hRIPΔN360*

Figure 4. Rev-directed RNAs accumulate on the outside of the nucleus in the presence of the *hRIPΔN360* mutant. Cos-1 cells were transfected with the indicated plasmids and RNA localization analyzed by fluorescent in situ hybridization as in the previous experiment (Fluorescence, left column) and differential interference contrast (DIC) microscopy (Nomarski, right panel). (Control panels) No DAPI, no probe; (DAPI) (+) DAPI; (Cy3) (+) probe; (merge) (+) DAPI and probe.

that *hRIPΔN360*'s ability to interfere with Rev function is not simply due to a general block in the transport of NLS- or NES-containing proteins.

We also examined whether *hRIPΔN360* could interfere with the general mRNA export pathway. HeLa cells

Figure 5. The hRIP Δ N360 mutant specifically inhibits Rev-directed RNA export. (A) The hRIP Δ N360 mutant does not affect the intracellular distribution of NLS- or NES-containing proteins or total poly(A)⁺ mRNA in mammalian cells. Cos-1 cells were transfected with the indicated EGFP-fusion reporter plasmid in the absence or presence of phRIP Δ N360 (*first* and *second* rows, *left* and *center* panels). HeLa cells were transfected with phRIP Δ N360 and the intracellular distribution of poly(A)⁺ mRNA was analyzed by in situ hybridization using an Alexa Green-labeled oligo dT₅₂ probe (*third* row, *left* and *center* panels). The intracellular distribution of hRIP Δ N360 was analyzed by indirect immunofluorescence microscopy using anti-HA (primary) and Cy3-labeled anti-mouse (secondary) antibodies (*right* panels). (B) The hRIP Δ N360 mutant is localized at the nuclear periphery in mammalian cells. Cos-1 cells were transfected with phRIP Δ N360 and analyzed by indirect immunofluorescence as in A (Fluorescence, *left* panel) and DIC microscopy (Nomarski, *center* and *right* panels). (C) The hRIP Δ N360 mutant does not affect the intracellular distribution of MLV envelope glycoproteins in mammalian cells. HeLa cells were transfected with an MLV-envelope expression plasmid (pSV-A-MLV-env) alone (*first* row) or cotransfected with phRIP Δ N360-EGFP (*second* row). The intracellular distribution of nascent MLV envelope glycoproteins was analyzed by indirect immunofluorescence microscopy using anti-MLV (primary) and anti-rat Alexa Red (secondary) antibodies. (D) The hRIP Δ N360 mutant does not affect constitutive secretion of SEAP in mammalian cells. 293-T cells were cotransfected with the indicated plasmid in the presence of pCMV β -gal. Untransfected cells were incubated with Brefeldin A (BFA) as a positive control for SEAP inhibition. Endogenous SEAP activity (*left* panel) and β -galactosidase expression (*right* panel) were monitored using standard enzyme assays. SEAP secretion levels were quantified as the ratio of SEAP activity in culture supernatants to the sum of SEAP activity in both the culture supernatants and cell lysates.

were transfected with pCMV or pHIPΔN360 in the presence of β-galactosidase expression plasmid (pCMV β-gal). The intracellular distribution of total cellular poly(A)⁺ mRNA was analyzed by in situ hybridization with a fluorochrome-labeled oligonucleotide dT₍₅₂₎ probe. In contrast to our findings for Rev-directed RNAs, there was no significant change in the intracellular distribution of poly(A)⁺ mRNA in the presence of hRIPΔN360 (Fig. 5A, third row, left and center panels). Similarly, the cytoplasmic accumulation of heat shock protein 70 (hsp70) mRNA or RNA containing the Mason–Pfizer monkey virus (MPMV) constitutive transport element (CTE) were also unaffected by hRIPΔN360 (data not shown). These results provide additional evidence that the nature of the inhibition exerted by hRIPΔN360 on Rev function is specific.

Our in situ hybridization assays revealed that the mislocalized Rev-directed RNAs have a similar intracellular distribution to that of hRIPΔN360 (Figs. 4 and 5B). Given hRIPΔN360's proximity to the endoplasmic reticulum (ER), Golgi apparatus, and vesicular structures of the endosome-lysosome system, we considered whether its intracellular location could affect cellular processes mediating vesicular transport and protein secretion. Disruption of such trafficking pathways might prevent movement of Rev-directed RNAs from the nuclear periphery to the cytoplasm. To explore this possibility, we tested whether hRIPΔN360 could affect the intracellular distribution of the murine leukemia virus envelope glycoprotein (MLV-Env), an integral membrane protein that uses cellular vesicular pathways for transport from the *trans* Golgi network (TGN) to the plasma membrane (Miller et al. 1994). HeLa cells were transfected with an MLV-Env expression plasmid (pSA-A-MLV-env; Landau et al. 1991) in the absence or presence of an hRIPΔN360-EGFP expression plasmid (pHIPΔN360-EGFP). Similar to the untagged derivative, hRIPΔN360-EGFP causes Rev-directed RNAs to mislocalize and aberrantly accumulate at the nuclear periphery of transfected cells (data not shown). The intracellular distribution of nascent MLV-Env proteins was analyzed using indirect immunofluorescence microscopy with anti-MLV-Env antibodies (Evans et al. 1990). Consistent with previous reports, the MLV-Env proteins were localized predominantly at the plasma membrane (Fig. 5C, first row). hRIPΔN360-EGFP has no discernible effect on the intracellular distribution of the MLV-Env proteins (Fig. 5C, second row). Importantly, the intracellular localization of hRIPΔN360-EGFP was spatially distinct from that of MLV-Env. These results further indicate that hRIPΔN360-GFP failed to disrupt the cytoplasmic accumulation and expression of MLV-env mRNAs.

As a complementary approach, we examined whether hRIPΔN360 could block an early step in the constitutive secretory pathway using a secreted alkaline phosphatase assay (SEAP). In brief, 293T cells, which secrete appreciable levels of SEAP (Peretea et al. 2003), were transfected with pCMV, pHIP, pHIPΔN360, or pEGFP in the presence of pCMV β-gal, and endogenous SEAP activity was monitored using a standard chromogenic en-

zyme assay (Liu et al. 2001). To control the specificity of inhibition, we treated mock transfected cells with Brefeldin A (BFA), a fungal toxin that selectively disrupts trafficking through the Golgi (Chardin and McCormick 1999). Figure 5D (left panel) shows the constitutive SEAP secretion in 293T was reduced following a mild BFA treatment. In contrast, SEAP levels were unchanged in cells expressing hRIP or the hRIPΔN360 mutant. The cytoplasmic accumulation and expression of the internal control β-galactosidase mRNA was also unaffected in the presence of hRIPΔN360 (Fig. 5D, right panel). Collectively, these results indicate that the perinuclear presence of hRIPΔN360 does not interfere with vesicular trafficking pathways used for intracellular distribution of integral membrane proteins or constitutive protein secretion. Thus, we conclude that the inhibition exerted by hRIPΔN360 on Rev-directed RNA export is specific.

Ablation of hRIP activity by RNA interference results in the loss of Rev function

To further substantiate the requirement for hRIP in Rev function, we examined the intracellular distribution of Rev-directed RNAs in cells depleted of endogenous hRIP by RNA interference (Elbashir et al. 2001, 2002). Mammalian cells were transfected with synthetic small interfering RNA duplexes (siRNAs) homologous to nucleotides 1593–1613, 1106–1126, or 463–483 of the hRIP coding sequence (hRIP-specific siRNA H1, H2, and H3, respectively). Alternatively, cells were transfected with siRNAs harboring a single base pair mismatch within the same hRIP sequences (mutant siRNA M1, M2, and M3) using Oligofectamine. Cellular hRIP levels were analyzed by Western blotting of cell extracts from the bulk culture using anti-hRIP polyclonal antibodies. The results in Figure 6A show that hRIP was efficiently depleted following treatment with H1 siRNAs, but unaffected by Oligofectamine alone (first row, lanes 1 and 2). Depletion of cellular hRIP was also achieved, albeit to a lesser extent, using H2 siRNAs (Fig. 6A, lane 3), whereas the H3 or M3 siRNA had no effect on cellular hRIP levels (data not shown). Importantly, transfection of the M1 or M2 siRNAs failed to reduce the level of cellular hRIP (Fig. 6A, lanes 4 and 5). Additionally, the level of endogenous control protein Lamin A/C was unchanged by any siRNA treatment, further demonstrating the RNA interference is specific for hRIP expression (Fig. 6A, second row). Using these optimized conditions, we tested whether ablation of hRIP activity could interfere with Rev function. Cells were treated with the hRIP-specific or mutant siRNAs, then cotransfected with pCMV128 and pcRev or pgTAT and pcRev. CAT activity and intracellular RNA localization were analyzed as in previous experiments. Figure 6B shows that hRIP-depleted cells failed to support Rev function in this assay. Moreover, we observed a dose-dependent correlation between the level of hRIP depletion and the loss of Rev function (Fig. 6B, lanes 2, 3, 5, and 6). Most important, in situ hybridization analysis revealed that Rev-directed RNAs were mislocalized and aberrantly accumulated at the nuclear

hRIP is an essential cellular cofactor for HIV Rev

Figure 6. Ablation of hRIP activity inhibits HIV Rev function by mislocalizing Rev-directed RNAs to the nuclear periphery. (A) Depletion of cellular hRIP using RNA interference. HeLa cells were transfected with hRIP-specific (H1 or H2) or mutant siRNA duplexes (M1 or M2) using Oligofectamine. Control cells were transfected with Oligofectamine alone (no siRNA). hRIP and Lamin A/C levels in cell lysates prepared from the bulk culture were analyzed by Western blotting using goat anti-hRIP or anti-Lamin A/C polyclonal antibodies, respectively, and visualized by ECL. (B) Ablation of hRIP activity inhibits Rev function in mammalian cells. HeLa cells treated with the indicated siRNA duplexes were cotransfected with pCMV128, pcREV, and pCMV β -gal. CAT and β -galactosidase enzyme activities were assayed as in Figure 2. (1X) 50 nM; (2X) 100 nM; (–) absence; (+) presence. (C) Rev-directed RNAs mislocalize and aberrantly accumulate at the nuclear periphery of hRIP-depleted cells. HeLa cells treated with siRNA M1 or H1 were cotransfected with pgTAT and pcRev and RNA localization analyzed as in Figure 3.

periphery in hRIP-depleted cells (Fig. 6C). Thus, the intracellular distribution of Rev-directed RNAs in hRIP-depleted cells was strikingly similar to that observed in the presence of the hRIP dominant-negative mutant. It is also worth noting that ablation of hRIP activity by RNA interference had no effect on the intracellular distribution of the HIV-based and cellular NLS- or NES-containing proteins or the internal control β -galactosidase mRNA (data not shown). Collectively, our results indicate that hRIP is an essential cellular cofactor for Rev-directed RNA export, which acts at a previously unanticipated step: movement of RNAs from the nuclear periphery to the cytoplasm.

Discussion

We have determined a function for the cellular HIV-1 Rev interacting protein, hRIP, during HIV-1 Rev-directed RNA export. Ablation of hRIP activity by either a *trans*-dominant-negative mutant or siRNAs inhibits HIV-1 Rev function by mislocalizing RRE-containing RNAs to the nuclear periphery. Comparable ablation of hRIP activity did not affect intracellular localization or trafficking of a variety of proteins or cellular poly(A)⁺ mRNA.

Thus, hRIP is an essential cellular cofactor for HIV-1 Rev function that acts by promoting movement of RRE-containing RNAs from the nuclear periphery to the cytoplasm.

Movement from the perinuclear region as a step in nuclear RNA export

Our results identify release from the nuclear periphery as an essential step in HIV-1 Rev-directed RNA nuclear export. Moreover, this perinuclear step may link nuclear export with cytoplasmic RNA localization and function. The RNA mislocalization phenotype resulting from hRIP inhibition is strikingly different from the strictly nuclear mislocalization previously observed due to inhibition of Rev mutants or CRM1. This mislocalization pattern was particularly unexpected because CRM1 is thought to bridge the interaction between Rev and hRIP, and therefore one would predict the pattern to resemble that of CRM1 inhibition. Furthermore, it is significant that this mislocalization phenotype requires both Rev and an RRE-containing RNA. This high degree of specificity verifies that this RNA mislocalization represents

a block in an intermediate step in Rev-directed RNA export.

The results from our localization studies with poly(A)⁺ mRNA suggest that hRIP is not a general mRNA export factor. Nevertheless, cellular RNAs would, presumably, have to undergo a similar perinuclear release step. We therefore predict there are proteins with analogous functions to hRIP which act on cellular mRNAs to promote their movement from the nuclear periphery to the cytoplasm.

An extended role for Rev

A role for the perinuclear region in Rev-directed RNA export is consistent with several previous findings. First, Rev is required for efficient translation of the intron-containing *gag-pol* and *env* RNAs (Arrigo and Chen 1991; Lawrence et al. 1991; D'Agostino et al. 1992; Favaro et al. 1999) and has been proposed to direct viral RNAs to the translation machinery (Kimura et al. 1996; Coyle et al. 2003). Second, the cellular protein SAM68 (src-associated in mitosis) can partially substitute for Rev in transient transfection assays (Reddy et al. 1999; Soros et al. 2001). Interestingly, a dominant-negative SAM68 mutant also mislocalizes Rev-directed RNAs to the nuclear periphery (Soros et al. 2001). Third, hRIP has been reported to interact with Eps15 (Doria et al. 1999), an EH-domain containing protein that is concentrated at the perinuclear region and can bind to CRM1 (Santolini et al. 1999; Vecchi et al. 2001).

Mechanism of hRIP action

Compared with our understanding of protein and RNA transport across the NPC, relatively little is known about the factors and mechanisms that facilitate release of RNAs from the cytoplasmic side of the NPC and their subsequent movement through the cytoplasm. Models for hRIP function must account for the finding that the mislocalization phenotype requires both Rev and an RRE-containing RNA. In addition, standard NES-containing protein substrates were not mislocalized in the absence of functional hRIP. Thus, hRIP is specifically involved in Rev-directed RNA localization.

One can invoke at least two models for how hRIP promotes movement of Rev-directed RNAs from the nuclear periphery into the cytoplasm. In the first model, hRIP could promote the disassembly of Rev-directed RNA from the export complex at the cytoplasmic side of the NPC. Several factors are known to be involved in disassembly of transport complexes following movement through the NPC, including RanBP1, RanBP2, and NXT1 (Kehlenbach et al. 1999; Black et al. 2001; Bednenko et al. 2003). If the function of hRIP were to remove Rev from its cognate RNA, failure to completely disassemble the export complex would result in retention of these RNAs at the nuclear periphery. Consistent with such a model, we note that hRIP contains a C₄H₂-type

zinc finger motif, which has been previously implicated in several activities, including the disassembly of macromolecular complexes (Spang 2002).

Alternatively, hRIP might function after disassembly of the export complex to facilitate the movement of Rev-directed RNA from the nuclear periphery into the cytoplasm. In this model, hRIP could act as a positive regulator for site-specific RNA localization within the cytoplasm. One pertinent example is Staufen, an RNA binding protein that has been implicated in the cytoplasmic trafficking of HIV-1 genomic RNA. Interestingly, like hRIP, Staufen is concentrated at the perinuclear region (Wickham et al. 1999), where HIV-1 RNAs are thought to be assembled into large Staufen-containing granules for subsequent trafficking out to the cell periphery (Mouland et al. 2000). Another example of a positive regulator is the zipcode binding protein 1 (ZBP1), which mediates actin-dependent localization of β -actin mRNA in chicken embryo fibroblasts (CEFs; for review, see Jansen 2001). Predominantly a cytoplasmic protein, ZBP1 binds to a 54-nt *cis*-acting element or "zipcode" located in the 3' UTR of the β -actin mRNA and colocalizes with the RNA to the leading lamellae (Farina et al. 2003). ZBP1 appears in large cytoplasmic granules, which codistribute with β -actin mRNA granules thought to contain components necessary for transport, localization, and translation of specific mRNAs. Based on these observations, it has been suggested that interaction of ZBP1 with the β -actin zipcode facilitates granule formation and cytoskeletal attachment, two processes that, ultimately, lead to the localization of the RNA.

Possible cellular function of hRIP

An important future objective will be to determine the cellular function of hRIP. Loss of hRIP activity does not affect the distribution of bulk poly(A)⁺ mRNA, indicating it does not play a general role in nuclear mRNA export. However, hRIP could act on a distinct subset of cellular mRNAs. In this regard, in several experiments, we observed small, discrete patches of RNA at the nuclear periphery of cells expressing the hRIP dominant-negative mutant. Perhaps these represent the subset of mRNAs whose cytoplasmic localization is mediated by a CRM1-dependent pathway (Brennan et al. 2000; Gallouzi and Steitz 2001).

hRIP can interact via CRM1 with nuclear export factor 3 (NXF3), a protein implicated as a factor involved in mRNA export (Herold et al. 2001; Yang et al. 2001). NXF3 is predominantly expressed in human testis and has been suggested to play a role in male germ cell development, perhaps as a tissue-specific nuclear RNA export factor. hRIP is also expressed at high levels in testis (Perteaux et al. 2003) and hRIP-deficient male mice are infertile, suggesting a role for hRIP during mouse spermatogenesis (Kang-Decker et al. 2001).

Regardless of the cellular role of hRIP, our results clearly show that it is required for the RNA transport function of HIV-1 Rev. It is well established that Rev is

essential for HIV-1 replication and that inhibition of Rev function interferes with virus production. These considerations raise the possibility that hRIP is a cellular cofactor required for HIV-1 replication and a potential target for new antiviral strategies.

Materials and methods

Plasmid construction

N- and C-terminal truncated hRIP cDNAs were generated by PCR amplification [hRIP nts (Accession No. X89478): 1–1530(Δ C52), 1–1260(Δ C142), 1–975(Δ C237), 1–735(Δ C317), 262–1686(Δ N87), 706–1686(Δ N235), 1081–1686(Δ N360), and 1267–1686(Δ N422)] and inserted in the HindIII/XbaI restriction sites of pCMV-HA-Flag (Roberts et al. 1995). phRIP Δ N360-EGFP contains nucleotides 1081–1686 of hRIP inserted into HindIII/BamHI restriction sites of pEGFP-N1 (Clontech). All recombinant expression plasmids were analyzed by DNA sequencing prior to individual transfection into Cos-1, HeLa, or 293-T cells. Protein levels in cell lysates (50–75 μ g total protein) were analyzed by Western blotting using anti-HA (12CA5 at 1:1200; BABCO) or anti-Flag (M5 at 1:1000; Sigma) monoclonal antibodies and visualized by enhanced chemiluminescence (ECL; Amersham). The mutant hRIP protein levels were comparable to full-length hRIP in these mammalian cell lines.

Cell lines and media

Monkey Cos-1, human HeLa, and 293-T fibroblasts were grown in DMEM (Life Technologies) supplemented with 25 mM Hepes (pH 7.2), 44 mM sodium bicarbonate, 11% fetal bovine serum (Clontech), 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C, 10% CO₂.

Transient transfection assays

Transient transfection assays of Rev function in Cos-1 cells were performed as described (Zapp et al. 1993). Transfection mixtures contained 0.5 μ g pCMV128 (Hope et al. 1990), 0.5 μ g pCMV128, and 1 μ g pcRev (Malim et al. 1989); 0.5 μ g pCMV128, 1 μ g pcRev, and 2.5 μ g phRIP; or 0.5 μ g pCMV128, 1 μ g pcRev, and 2.5 μ g phRIP Δ N235, phRIP Δ N360, or phRIP Δ C237. Additional transfection mixtures contained 0.5 μ g pCMV128, 1 μ g pcRev, and 0 to 8 μ g of phRIP Δ N360. All transfection mixtures contained 0.2 μ g pCMV β -gal (Clontech), a β -galactosidase expression plasmid as an internal control for transfection efficiency, protein normalization, and nonspecific effects of the CMV promoter. Total DNA was adjusted to 10 μ g using pBC-IL2 (Cullen 1986), pCMV-HA-Flag, and pUC118. All CAT assays were normalized to the expression of β -galactosidase activity as described in Hope et al. (1990). Transient transfection assays were performed in duplicate, with the data shown in Figure 2 representing six independent experiments.

In situ hybridization assays

Cos-1 cells were incubated with the transfection mixture for 8 to 12 h, washed with PBS (1 mM KH₂PO₄, 10 mM Na₂HPO₄, 130 mM NaCl, 2.7 mM KCl at pH 7.0), then re-fed with 3 mL DMEM. Transfection mixtures contained 1 μ g pgTAT; 1 μ g pgTAT and 2.5 μ g pcRev; or 1 μ g pgTAT, 2.5 μ g pcRev, and 4 μ g phRIP or phRIP Δ N360. Control mixtures contained 1 μ g pgTAT and 2.5 μ g pcRev M10, or 1 μ g pgTAT and 3 μ g pCMV Δ CAN.

Total DNA was adjusted to 10 μ g using pBC-IL2, pCMV, and pUC118. At 18 to 22 h posttransfection, cells were treated with 0.5% trypsin/0.1 mM EDTA, and resuspended in 5 mL media, plated onto three to five sterile glass coverslips in 60-mm dishes containing 2.5 mL of media, and incubated further for 48–72 h. Cells were then washed in PBS, incubated in fixation solution (4% EM-grade paraformaldehyde [EMS], 1 $\times$ PBS, 5 mM MgCl₂) for 15 min, rinsed, and stored in 70% ethanol at 4°C until use. Hybridization reactions were performed in triplicate as described previously (Zhang et al. 1996) and contained 60–120 ng of labeled probe. Following a posthybridization step, coverslips were mounted on glass slides using 10 μ L Vectashield containing 10 μ g/mL DAPI (Vector Laboratories). Hybridization signals were visualized using a Zeiss Axioplan 2 fluorescence microscope equipped with a 63 $\times$ plan Apochromat objective. Images were acquired and analyzed using Open Lab 2.2.5 software.

Oligonucleotide probes

In situ oligonucleotide probes were labeled with Cy3 (Amersham) or Alexa Green 488 (Molecular Probes) according to the manufacturer's instructions. The RRE-specific probe is complementary to nucleotides 7353 through 7420 of the HIV-1_{LAI} RNA (Accession No. K03455). The probe used to detect cellular poly(A)⁺ mRNA consisted of 52 thymidine residues (dT₅₂).

Immunofluorescence analysis

HIV-NLS-and NES-EGFP reporter proteins Cos-1 cells were transfected with pHIV-NLS-EGFP or pHIV-NES-EGFP (1–2 μ g) alone or in the presence of phRIP Δ N360 (4 μ g) as described in Bartz et al. (1996). After 12 h, the transfection mixture was removed, and the cells washed twice with PBS. Following treatment with 0.5% trypsin/0.1 mM EDTA, cells were suspended in 1 mL media and plated onto three to five sterile coverslips in 60-mm dishes. After 30 min at 37°C, 2 mL media was added to each dish, and the cells were incubated further for 24–48 h. For analysis, cells were incubated in ice-cold methanol for 7 min, then treated with cytoskeletal (CSK) buffer (10 mM PIPES at pH 6.9, 100 mM KCl, 300 mM sucrose, 5 mM MgCl₂, 2 mM EGTA, 0.5% Triton-X-100) for 10 min at 25°C. Cells were blocked in Buffer B (0.2% bovine serum albumin, PBS, 0.1% Triton-X-100) for 30 min, then incubated in Buffer B containing an anti-HA antibody (at 1:500) for 1 h. Following washing in PBS, cells were incubated in Buffer B containing affinity-purified donkey anti-mouse Cy3-labeled antibodies (at 1:750; Kirkegaard and Perry Laboratories). After 30 min, the cells were washed in PBS and mounted as described for in situ hybridization.

MLV envelope proteins HeLa cells grown to 50% confluence were cotransfected with pSV-A-MLV-env (5 μ g) and pCMV (5 μ g), pSV-A-MLV-env (5 μ g) and phRIP Δ N360-EGFP (5 μ g), or pCMV (10 μ g) using the Calcium Phosphate Transfection System (Life Technologies). At 12 h posttransfection, cells were processed as described in the previous section using an anti-MLV envelope rat monoclonal antibody (83A25, Evans et al. 1990, primary at 1:2000) and an anti-rat Alexa Red 594 (Molecular Probes, secondary at 1:5000).

SEAP assays 293T cells were transfected with pCMV, phRIP, phRIP Δ N360, or pEGFP expression plasmids (5 μ g) as described by Bartz et al. (1996). Duplicate transfection reactions also contained pCMV β -gal (0.2 μ g) as an internal control for transfection efficiency and protein normalization. Control untransfected cells were treated with BFA (2.5 μ g/mL, Sigma) as described previously (Ding et al. 2001). Cell viability (>99.4%) was monitored by trypan blue staining following DNA transfection.

or BFA treatment. Cells and culture supernatants were harvested at 48 and 72 h posttransfection and SEAP activity assays were performed as described in Liu et al. (2001). The level of SEAP secretion was quantified as the ratio of SEAP activity in culture supernatants to the sum of SEAP activity in both the culture supernatants and cell lysates. Data shown in Figure 5D are representative of four independent experiments.

Small interfering RNA (siRNA)

Synthetic 21-nt RNA duplexes with symmetric 2-nt 3' (2'-deoxy) thymidine overhangs correspond to nts 1593–1613, 1106–1126, or 463–483 of the hRIP mRNA, which are unique in the human genome database. hRIP siRNA duplex sequences written in the 5' to 3' direction are as follows: (H1) CAGCCCAAUGGUGCA GGUUTT, AACCUGCACCAUUGGGCUGTT; (M1) GAGCC CAAUCGUGCAGGUUTTGT, AACCUGCACGAUUGGGC UGTT; (H2) CUGGCUUUGGGACCACAGGTT, CCUGUGG UCCCAAAGCCACTT; (M2) CUGGCUUCGGGACCACAG GTT, CCUGUGGUCCCGAAGCCACTT; (H3) GCCAAAGU CCUGGCAUCAGTT, CUGAUGCCAGGACUUGGCTT; (M3) GCCAAAGUCCUGCGAUCAGTT, CUGAUGCGAGGACUUU GGCTT. Oligonucleotides were annealed as described in Elbashir et al. (2001) and duplex formation was monitored using nonde-naturing gel electrophoresis and ethidium bromide staining.

siRNAs transfections

HeLa cells grown to 50% confluence in 60-mm dishes were transfected with hRIP-specific siRNA duplexes (50–100 nM) using Oligofectamine as described previously (Elbashir et al. 2002). At 16–20 h posttransfection, cells were retransfected with 1.4 µg pCMV128, 3 µg pcRev, and 0.2 µg pCMV β or 1 µg pgTAT and 2.5 µg pcRev using Gene-PORTER (GTS). Cell viability (>98.7%) was monitored by trypan blue staining following siRNA treatment and DNA transfection. Transfected cells were harvested after 48–72 h at 37°C, suspended in 450 µL RIPA buffer (10 mM Tris-HCl at pH 7.4, 1 mM EDTA, 0.1% SDS, 1% TX-100, 1% sodium deoxycholate, 150 mM NaCl, and 0.2 mM PMSF), incubated for 30 min on ice, then centrifuged at 13,000g for 15 min at 4°C. Supernatants (lysates) were quantified using Total Protein Reagent (Sigma), and equal amounts of protein (25–50 µg) resolved on a 10% SDS denaturing polyacrylamide gel (National Diagnostics). Protein compositions were analyzed by Western blotting using goat anti-hRIP (Santa Cruz Biochemical, C-19 at 1:750; goat anti-Lamin A/C, N-18 at 1:500) and visualized by ECL/Amersham. CAT activity assays and RNA in situ hybridization analysis using hRIP-depleted cells were performed as described in a previous section.

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hRIP, a cellular cofactor for Rev function, promotes release of HIV RNAs from the perinuclear region

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