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Virology 330 (2004) 471–480

VIROLOGY

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A DEAD box protein facilitates HIV-1 replication as a cellular co-factor of Rev

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Received 1 September 2004; returned to author for revision 21 September 2004; accepted 25 September 2004

Abstract

HIV-1 Rev escorts unspliced viral mRNAs out of the nucleus of infected cells, which allows formation of infectious HIV-1 virions. We have identified a putative DEAD box (Asp–Glu–Ala–Asp) RNA helicase, DDX1, as a cellular co-factor of Rev, through yeast and mammalian two-hybrid systems using the N-terminal motif of Rev as "bait". DDX1 is not a functional homolog of HIV-1 Rev, but down-regulation of DDX1 resulted in an alternative splicing pattern of Rev-responsive element (RRE)-containing mRNA, and attenuation of Gag p24 antigen production from HL*fb rev*(−) cells rescued by exogenous Rev. Co-transfection of a DDX1 expression vector with HIV-1 significantly increased viral production. DDX1 binding to Rev, as well as to the RRE, strongly suggest that DDX1 affects Rev function through the Rev–RRE axis. Moreover, down-regulation of DDX1 altered the steady state subcellular distribution of Rev, from nuclear/nucleolar to cytoplasmic dominance. These findings indicate that DDX1 is a critical cellular co-factor for Rev function, which maintains the proper subcellular distribution of this lentiviral regulatory protein. Therefore, alterations in DDX1–Rev interactions could induce HIV-1 persistence and targeting DDX1 may lead to rationally designed and novel anti-HIV-1 strategies and therapeutics.

Keywords: HIV-1; Rev; DDX1; RNA Helicase; DEAD Box; Co-factor

Background

Rev is one of the early gene products expressed during the human immunodeficiency virus type I (HIV-1) replication cycle. After viral entry into host cells, Rev is expressed from fully spliced viral mRNA, as also observed for the viral regulatory proteins, Tat and Nef (Hope and Pomerantz, 1995; Kjems and Askjaer, 2000; Pollard and Malim, 1998). Wild-type Rev expression is required for generation of infectious HIV-1 virions (Hope and Pomerantz, 1995; Feinberg et al., 1986). Hence, expression of Rev below a threshold level may be responsible for some mechanisms of cellular persistence or latency of HIV-1 infection (Pomerantz et al., 1992). Wild-type Rev enables the unspliced and single-spliced viral mRNAs to be exported to the cytoplasm, where viral structural proteins can be translated and genomic viral RNA may be encapsidated. The efficient nuclear/cytoplasmic RNA trafficking is accomplished by a cognate association between Rev and the Rev-responsive element (RRE) within the target lentiviral RNA, on the basis of multiple interactions with cellular co-factors.

To date, two major Rev domains, a nuclear/nucleolar localization signal (NLS) and a nuclear export signal (NES), have been investigated and several co-factors were identi-

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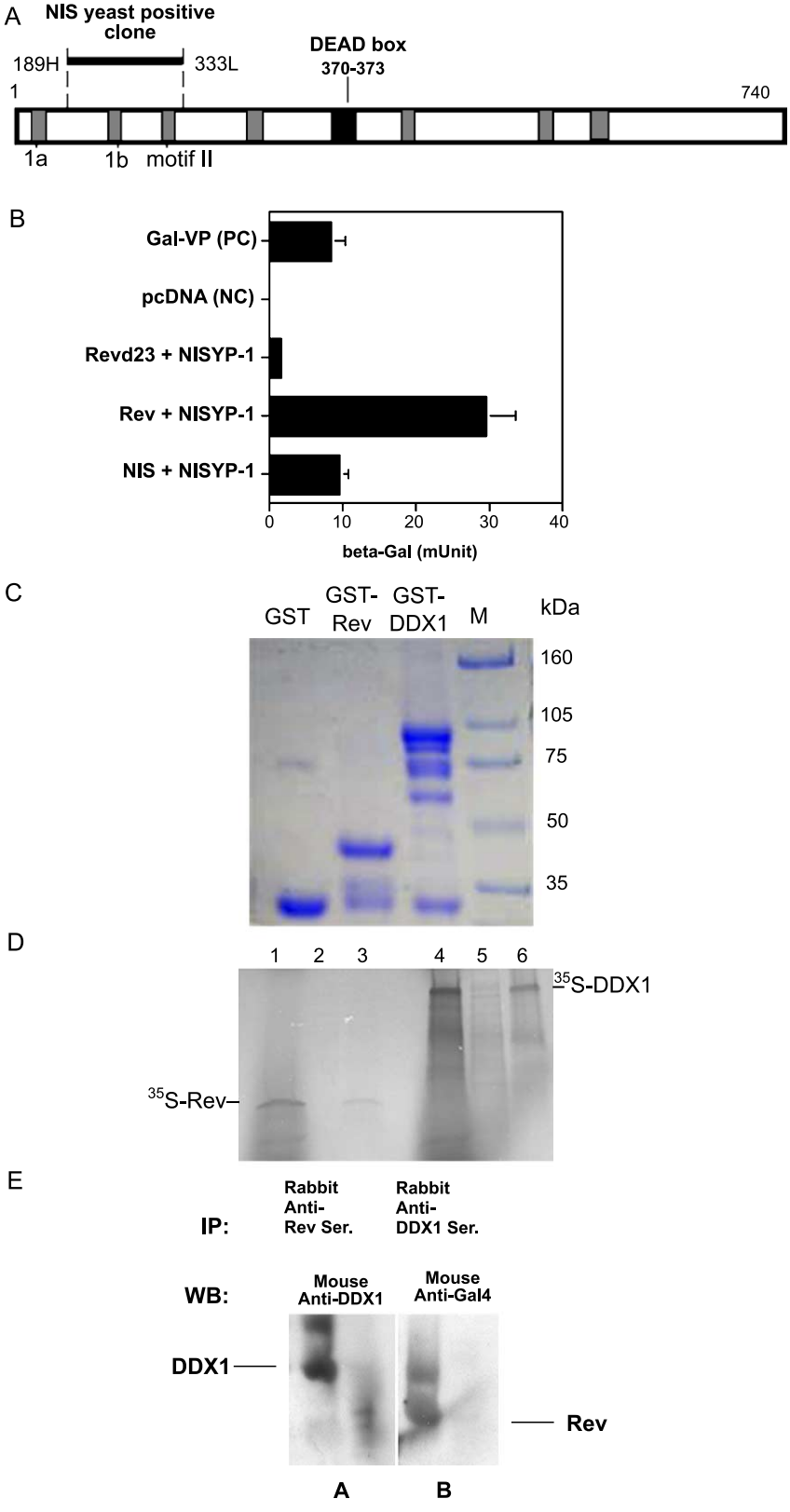
doi:10.1016/j.virol.2004.09.039

fied for these motifs, importantly including CRM1 (exportin) for the NES and β-importin for the NLS (Pollard and Malim, 1998). In addition, the N-terminus has also been found to be crucial for Rev function, as certain deletions or mutations in the region resulted in diminished or absent Rev activity (Daly et al., 1995; Hope et al., 1990; Kubota and Pomerantz, 1998, 2000; Malim et al., 1989; Van Ryk and Venkatesan, 1999). Such mutants are characterized by: (1)



loss of specific binding to the RRE; (2) failure in exporting intron-containing viral transcripts from the nucleus; (3) loss of dominant nuclear/nucleolar localization; (4) inefficient multimerization of Rev; and (5) a dominant-negative phenotype (Fang et al., 2002). A small domain with 15 amino acid residues in the N-terminus of HIV-1 Rev was identified by our group as a nuclear diffusion inhibitory signal (NIS; Kubota and Pomerantz, 1998, 2000), which is required to maintain the nuclear dominant subcellular distribution and function of Rev. We hypothesized that such effects of NIS in Rev would likely be accomplished by the association between the Rev NIS and specific cellular co-factor(s). We now report on the identification of a novel DEAD box protein, as a counterpart of the Rev NIS, which is demonstrated to be a critical cellular co-factor supporting HIV-1 replication.

Results

Association of HIV-1 Rev with DDX1, a putative RNA helicase

By using NIS (Rev 10–24: EDLLKAVRLIKFLYQ) as "bait", we identified five clones showing interactions with NIS, through yeast two-hybrid screening of a cDNA library from the human Jurkat CD4+ T-cell-line. Nucleotide sequence determination and analysis revealed that two of the five positive clones were identical, and represented partial cDNAs from the human DDX1 gene (NM-004939), encoding 145 aa of this full-length, 740 aa nuclear-localized DEAD box protein (Godbout and Squire, 1993). DEAD box proteins contain seven conserved domains and are considered to be putative RNA helicases (Fig. 1A; Godbout and Squire, 1993; Godbout et al., 1998).

The association of Rev and a fragment of the DDX1 gene product (189 H to 333L; NISYP-1 for NIS yeast positive clone 1) was then confirmed in a mammalian two-hybrid system (Fig. 1B). The 15-residue NIS of HIV-1 Rev demonstrated a strongly positive signal with the DDX1 fragment, indicating the association of NIS and DDX1. Interestingly, wild-type Rev (as a Gal–Rev fusion protein) yielded an approximately three-fold stronger signal than that of NIS alone, indicating stable interactions of DDX1 with wild-type Rev, which is present in HIV-1-infected cells. As expected, in contrast to the wild-type, a NIS-deficient Rev mutant, Revd23 (Fang et al., 2002), showed no significant signal, indicating loss of association with DDX1 (Fig. 1B).

We further confirmed the association between full-length Rev and DDX1 with both in vitro and in vivo experiments (Figs. 1C, D, and E). In vitro GST pull-down assays showed significant binding between DDX1 and the GST–Rev fusion protein (Fig. 1D, lane 6), and a somewhat weaker but clearly demonstrable signal of Rev binding to the GST–DDX1 fusion protein (Fig. 1D, lane 3). Co-immunoprecipitation assays confirmed that the two over-expressed proteins bound to each other, revealing an in vivo physiological interaction in HEK293 cells. Of note, anti-Rev antibodies used for immunoprecipitation with anti-DDX1 for Western blotting, as well as the reciprocal, both demonstrated this intracellular protein–protein interaction (Fig. 1E).

DDX1 is required for the efficient function of HIV-1 Rev

We next asked whether DDX1 could function analogously to Rev, in facilitating the export of intron-containing mRNA, also containing the RRE, from the nucleus. A reporter vector for Rev function, pDM628, was used in the initial assays. In this system, exogenously expressed Rev protein binds to RRE on the unspliced reporter mRNA, allowing its export out of the nucleus for luciferase reporter gene expression. Co-transfection of Cos-1 cells with pDM628 and pcDDX1 yielded a signal only modestly higher than background (Table 1). Considering the difference that molar quantities of DNA (DDX1: 2.7 kb, Rev: 0.6 kb) may have on the signal levels, we repeated the experiments by using higher quantities of pcDDX1, and observed only approximately a 10% increased signal (data not shown). We concluded that DDX1 alone is not a potent Rev functional homolog, but may still alter Rev function.

We then tested, in the same system, the possibility that DDX1 is required directly for the proper functioning of Rev. DDX1 has been found ubiquitously expressed in many human cell-types (Godbout et al., 1998, and our unpublished observations), where it is available for HIV-1 replication, with various efficiencies. We utilized, in a complementary

Fig. 1. Association between DDX1 and HIV-1 Rev. (A) The full-length DDX1 molecule, with schematic illustration of its domains and the position of the DDX1 segment interacting with the NIS signal of HIV-1 Rev. (B) In vivo interaction of DDX1 and NIS in mammalian cells. In total, 1.0 μg of normalized plasmid DNA was transfected into Cos-1 cells in six-well plates. The reporter plasmid, pGBβGal (0.5 μg), was used in combination with: (1) pSG5GAL-VP (PC: positive control), (2) pcDNA3.0 (NC: negative control), (3) pSG5GAL-Revd23 + pSG5NISYP-1-VP (189-333; DDX1–VP16 fusion protein expression vector), (4) pSG5GAL-Rev + pSG5NISYP-1-VP, or (5) pSG5Gal-NIS + pSG5NISYP-1-VP. The results represent mean (columns) and SD (standard deviations with error bars) from triplicate samples. (C) Coomassie blue staining of GST, GST-Rev and GST–DDX1 fusion proteins on a 4–20% SDS-PAGE gel. M-molecular marker lane. (D) GST pull-down assay illustrating in vitro DDX1-Rev binding. ^{35}S-Rev and ^{35}S-DDX1 were approximately 17 and 90 kDa, respectively. Lane 1, ^{35}S-Rev input; lane 2, ^{35}S-Rev + GST; lane 3, ^{35}S-Rev + GST-DDX1; lane 4, ^{35}S-DDX1 input; lane 5, ^{35}S-DDX1 + GST, and lane 6, ^{35}S-DDX1 + GST-Rev. (E) Co-immunoprecipitation demonstrates in vivo DDX1-Rev interaction. For specific procedures, see Materials and methods. WB: Western blot; IP: immunoprecipitation; Ser: Serum.

Table 1
Rev-dependant reporter gene expression assay

Plasmid	Relative luciferase units (RLU/s) ± SD
pDM628 only	439.7 ± 57.6
+pcRev	3483.0 ± 712.0
+pcDDX1	591.0 ± 201.7

Cos-1 cells were transfected with pDM628 (0.1 μg) + pcDNA3.0 (0.4 μg), pDM628 (0.1 μg) + pcRev (0.4 μg), and pDM628 (0.1 μg) + pcDDX1 (0.4 μg), respectively, in 12-well plates. The cells were lysed 24 h after infection for luciferase activity assays. The mean and standard deviation (SD) were from triplicate results for each sample.

fashion, both an antisense RNA to DDX1 (As-DDX1) approach, as well as RNA interference and designed small interfering RNAs (siRNA) for down-regulating DDX1 expression levels (siDDX1 used as an abbreviation). Efficient down-regulation of DDX1 was confirmed by immunofluorescence analyses (IFA) in pAs-DDX1 transfected HEK293 cells (Fig. 2C), but not in the control cells (i.e., non-transfected and promoter-only (phu6p-transfected cells; Figs. 2A and B), and neither the antisense nor siRNA constructs altered cell viability or proliferation (not illustrated). This target sequence was then used for synthesis of an siDDX1 duplex. Down-regulation by specific siRNA and antisense moieties to DDX1 was also confirmed by Western blotting (Fig. 2D). siDDX1, as well as pAs-DDX1, were transfected into Cos-1 cells. We observed that while antisense to DDX1 had significant potent inhibitory effects on Rev function, siDDX1 had an even more robust inhibitory effect on Rev function, at a concentration of 0.4 μM (Table 2). This may be hypothesized to be secondary to a longer

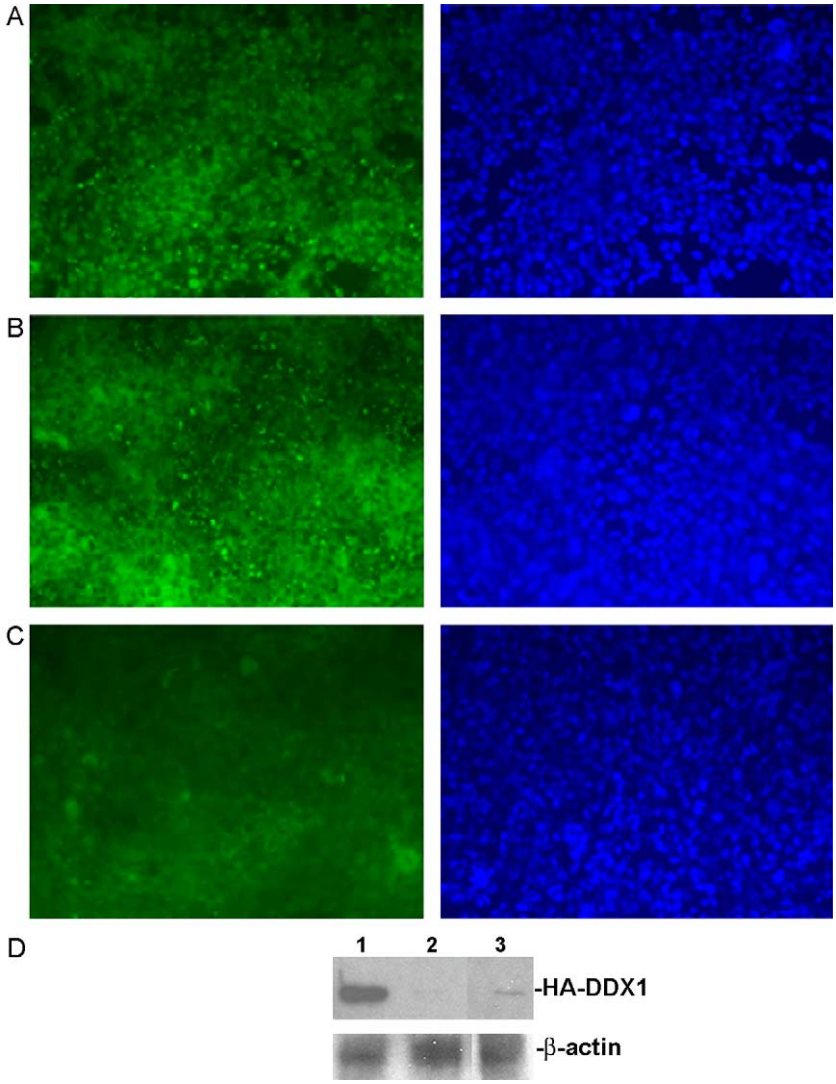


Fig. 2. Down-regulation of DDX1 in HEK293 cells. HEK293 cells were transfected with pcDDX1, under G418 selection for 2 weeks, followed by either no further transfection (A), transfection with phU6P (0.25 μg) (B), or pAS-DDX1 (0.25 μg) (C). The cells were treated 48 h later, by fixing and incubation with rabbit anti-human DDX1 antibody followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody. Right panels illustrate DAPI staining. The wide-field photomicrographs were obtained with a magnification of 10×, utilizing a Zeiss immunofluorescence microscope. The fields in the figure were representative of at least three fields selected randomly. (D) Western blotting analysis of specific down-regulation of DDX1 expression in HEK293 cells, by antisense DDX1 RNA and siRNA DDX1. (1) pCMV-HA-DDX1 only; (2) pCMV-HA-DDX1 + pAs-DDX1, and (3) pCMV-HA-DDX1 + siRNA DDX1.

Table 2
Inhibitory effects of siDDX1 and antisense DDX1 on Rev function

Plasmid	RLU/s ± SD
pDM628 only	901.3 ± 463.1
+pcRev	3446.1 ± 1,239.1
+pcRev + pAs-DDX1	1490.7 ± 324.5
+pcRev + siDDX1	61.2 ± 12.0

Cos-1 cells were transfected with pDM628 (0.1 μg), along with pcDNA3.0 (0.4 μg), pcRev (0.4 μg), pcRev (0.4 μg) + siRNA DDX1 (0.04 μM), and pcRev (0.4 μg) + pAs-DDX1 (0.2 μg), respectively, in a 12-well plate. The cells were lysed 24 h after transfection for luciferase activity assays. The mean and SD were from triplicate results for each sample.

time-period of intracellular activity of siRNAs, as compared to the larger size antisense.

We then confirmed the effects of antisense DDX1 and siDDX1 in HEK293 cells by achieving similar results as those described above in Cos-1 cells (data not illustrated), and further analyzed splicing pattern changes of mRNA from the RRE-containing reporter plasmid, pDM628, which reflects the effects on Rev function. Reverse transcriptase-polymerase chain reactions (RT-PCR) demonstrated a decreased ratio of unspliced RNA in cytoplasmic versus nuclear fractions (Fig. 3, first and third row), and an increased ratio of spliced versus unspliced RNA in the nuclear fraction (Fig. 3, second and third row), which supported the hypothesis that both pAs-DDX1 and siDDX1 restricted Rev function. The effects of antisense DDX1 and siDDX1 may also extend to specific cellular mRNA, as we observed weakened bands for certain house-keeping mRNAs (Fig. 3, bottom row).

We extended these studies to analyze HIV-1 replication. The production of HIV-1 p24 antigen can be restored by introducing exogenous Rev into a HLfb *rev*(−) cell-line, which contains an integrated, *rev*-deficient HIV-1 provirus (Mermer et al., 1990). We used this p24 antigen rescue assay to investigate the effects of down-regulation of DDX1 on

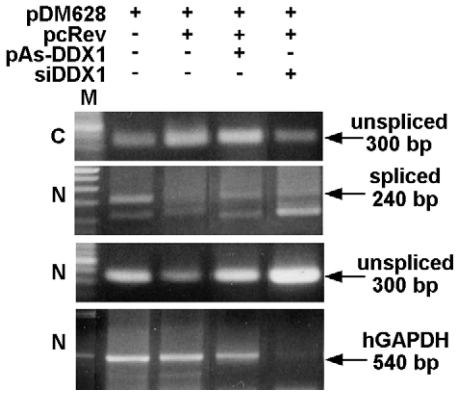


Fig. 3. Alteration of splicing patterns of RRE-containing mRNA by antisense DDX1 and siRNA DDX1. A total quantity of 1.5 μg DNA was utilized for transfection of HEK293 cells in each well of a six-well plate, including: pDM628 (0.2 μg) alone, pDM628 with pcRev (0.3 μg), pDM628 with pcRev and pAs-DDX1 (0.6 μg), and pDM628, pcRev plus siDDX1 (0.3 μM), prior to RT-PCR. M: DNA molecular marker; C: cytoplasmic fraction; N: nuclear fraction.

Rev function. Transfection of siRNA to DDX1 with wild-type Rev significantly decreased p24 antigen production (Fig. 4A). These data in toto demonstrated that a lower DDX1 level limits Rev function, both in Rev-dependant reporter expression and in Rev-dependant HIV-1 virion production. Since DDX1 expression is found at various levels in different cell-types, it is instructive to also

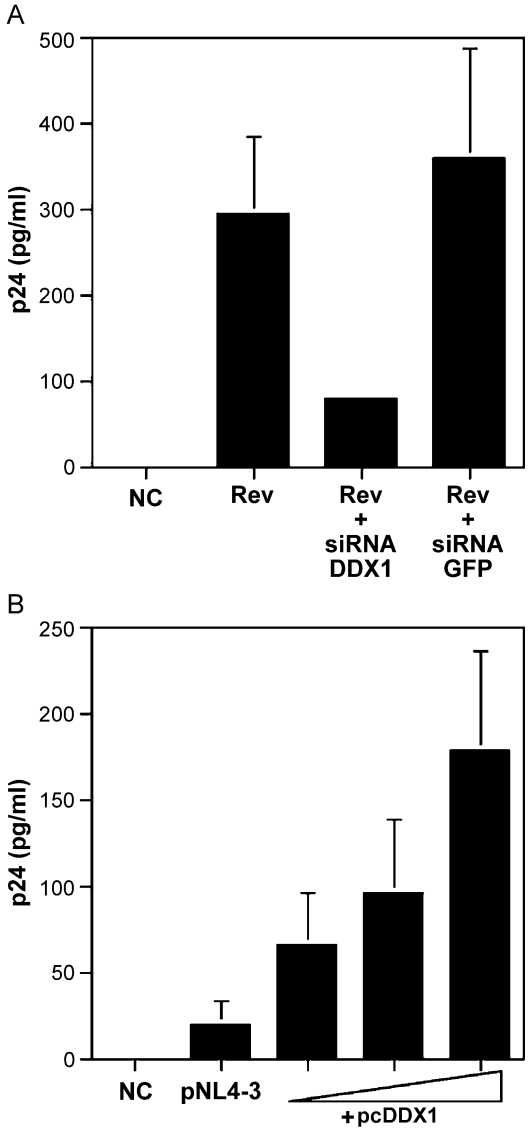


Fig. 4. Effects of DDX1 on HIV-1 replication. Inhibition of HIV-1 p24 antigen rescue by siRNA to DDX1. HLfb *rev*(−) cells seeded in 24-well plates (1×10^5 cells/well) were transfected with pcRev (0.2 μg) alone, pcRev with siRNA to DDX1, or pcRev with siRNA to GFP, at a siRNA concentration of 0.04 μM. Forty-eight hours post-transfection, the supernatants were collected for HIV-1 p24 antigen assay. The results are from triplicate samples. Augmentation of HIV-1 viral production by over-expressed DDX1. HEK293 cells in 24-well plates were transfected with pHIV-1-NL4-3 (33.3 ng) and differing quantities of pcDDX1 (30, 150 and 270 ng) per well. Total DNA quantities in each aliquot were standardized to be 0.33 μg, supplemented with pcDNA3.0. The supernatants were collected 48 h post-transfection for p24 antigen enzyme-linked immunosorbant assays (ELISA). The results are mean values with standard deviations (SD) from triplicate samples.

investigate effects on HIV-1 replication with DDX1 over-expression. The results showed that over-expression of DDX1 with very low quantities of transfected HIV-1_{NL4-3}, significantly augmented HIV-1_{NL4-3} replication (Fig. 4B). These results demonstrate that cellular DDX1 is required for proper Rev function, as well as for wild-type HIV-1 replication.

DDX1 is required for proper subcellular localization of HIV-1 Rev

As nuclear/nucleolar localization of Rev is required for proper function of this lentiviral regulatory protein, we analyzed effects of low DDX1 levels on Rev localization, preliminary to Rev functional assays. The rationale was that DDX1, as a NIS interacting protein, should play role(s) in Rev subcellular compartmentalization with intact NIS. Just as NIS-deficient Rev (Revd23) lost its proper localization and function, low cellular DDX1 levels should also alter localization and function of Rev, despite NIS integrity. After confirming efficient down-regulation of DDX1 (see above), co-transfection of pcRev with pAs-DDX1 in mammalian cells resulted in clear changes in Rev localization. While pcRev alone revealed expected nuclear/nucleolar dominance of wild-type Rev (Fig. 5A), combination of pcRev and pAs-DDX1 lead to a loss of nuclear/nucleolar dominant localization (Fig. 5B), and cytoplasmic dominance with lower quantities of nucleolar localization (Fig. 5C). No clear differences in Rev levels were detected, secondary to DDX1 expression. Therefore, we conclude that low DDX1 expression contributes to disordered Rev subcellular localization, which would likely alter Rev function and subsequent HIV-1 replication.

Effects of DDX1 on HIV-1 replication occur via the Rev–RRE axis

Further studies then evaluated whether DDX1 affects Rev function through Rev-RRE binding, a critical step generating unspliced HIV-1 genomic RNA delivery from the nucleus. Specific binding of DDX1 to RRE was assayed,

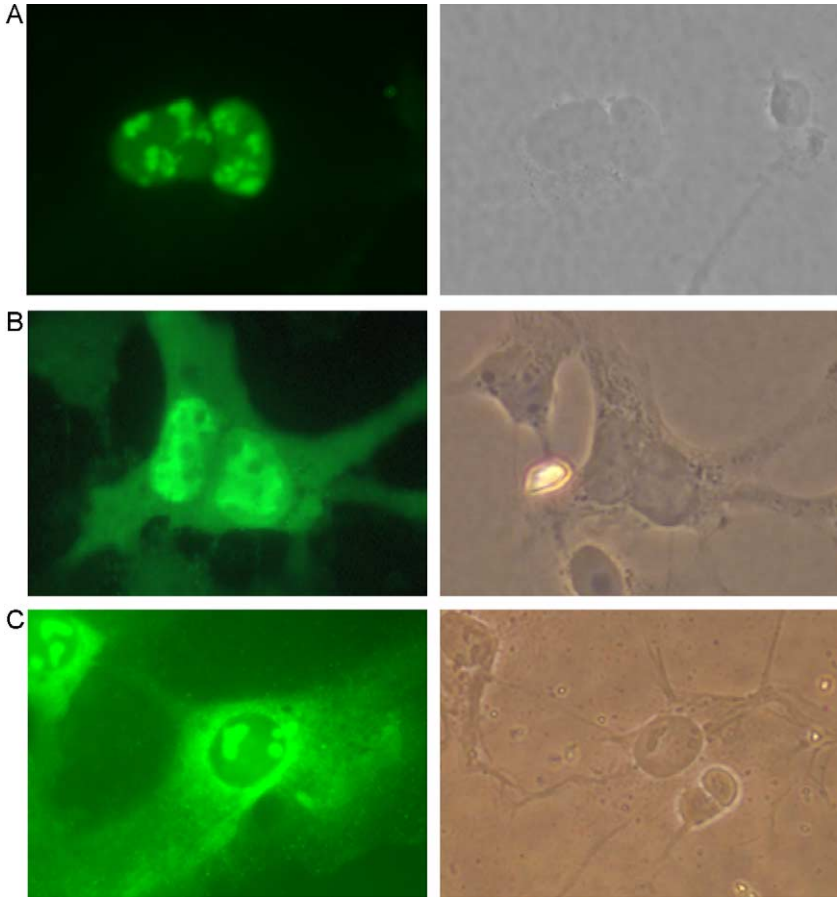


Fig. 5. Aberrant subcellular distribution of Rev in DDX-1-down-regulated Cos-1 cells. Cos-1 transient transfectants with a parental plasmid (A) or an antisense DDX1 expression plasmid (B and C) were co-transfected with pcRev (1 μg). The cells were fixed 48 h later and incubated in the rabbit anti-Rev polyclonal antibody, at a dilution of 1:200, followed by detection via a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody. The photomicrographs were obtained at a magnification of 40×. Two typical views of the subcellular distribution of Rev in DDX1-down-regulated cells are illustrated. The right panels are phase-contrast views of the same fields. These cells were analyzed via fluorescence microscopy. The photomicrographs were obtained at a magnification of 20×.

Table 3
In vivo RRE binding assay

Plasmids	RLU/s ± SD
pSLIIBLuc only	303.3 ± 79.1
+pTat/DDX1	1394.0 ± 105.7
+pTat/DDX1 + pcRev	184.0 ± 50.3
+pTat/DDX1 + pcDDX1	840.3 ± 304.7
+pTat/Rev	1561.3 ± 178.2
+pTat/Rev + pcRev	577.7 ± 246.4
+pTat/Rev + pcDDX1	1683.7 ± 307.2

Cos-1 cells (1.0×10^5), in a 12-well plate, were transfected with pSLIIBLuc (0.1 μg) and the indicated combination of plasmids. The cells were lysed 24 h after transfection for luciferase activity assays. The mean and SD were from triplicate results for each sample.

by using a luciferase reporter assay system in which luciferase gene expression depends on specific binding between a Tat–Rev fusion protein and the RRE. In this experiment, Tat–Rev was replaced by Tat–DDX1, and luciferase gene expression reflects specific RRE binding. Studies of Tat–DDX1 and Tat–Rev showed a similar luciferase activity, indicating that DDX1 is capable of RRE binding in vivo (Table 3). In this system, addition of pcRev, for expressing the cognate RRE-binding molecule and as a competitor of the Tat/DDX1 fusion protein, showed a significantly inhibited signal, thus indicating specific binding between DDX1 and the RRE. However, the magnitude of binding between DDX1 and RRE appears less robust than between Rev and RRE (Table 3), and DDX1 alone cannot rescue HIV-1 virion production from a Rev-negative provirus in the absence of Rev (data not illustrated). The binding of DDX1 to the RRE is, thus, compared to Rev binding to the RRE, significantly weaker. These results suggest that DDX1 binding to RRE, as well as to Rev, may render Rev capable of further interactions in the HIV-1 life-cycle.

Discussion

In this report, we demonstrate the requirement of DDX1 for efficient HIV-1 Rev function, critical roles for DDX1 in the Rev–RRE axis, and the contribution low-levels of DDX1 have on altering Rev localization. These data, for the first time, show the importance of the DEAD box protein, DDX1, in wild-type HIV-1 Rev function.

The NIS-binding protein, DDX1, clearly alters Rev function in a divergent manner, as compared to other important cellular co-factors of Rev that bind to the NLS or NES (Kjems and Askjaer, 2000). However, DDX1, may share some characteristics with Sam68, a member of heteronuclear ribonucleoprotein particle K (hnRNPK) homology (KH) domain family, as described in certain although not all articles on this (Li et al., 2002a,b; Lukong and Richard, 2003; Reddy et al., 1999, 2000). These include: (1) binding to Rev and the RRE, (2) low-levels of both factors (Sam68 and DDX1) restrict HIV-1 replication, and (3) over-expression of the factors boost HIV-1 replication. Sam68 enhanced unspliced RNA (including RRE-containing RNA) export, via pathways including CRM-1-dependant processes. Nonetheless, data from the present report and others show that DDX1 is functionally distinguishable from all descriptions of Sam68, which suggests potential unique significance for DDX1 (Bleoo et al., 2001; Chen et al., 2002). Of note, (1) DDX1 is not a Rev homolog but a factor binding to the unique pathophysiological NIS-motif and suggests that DDX1 affects Rev by maintaining spatial structure; (2) DDX1 has RNA helicase and ATPase activities; (3) DDX1 is usually located in both the nucleoplasm and within cleavage bodies of the nucleus in which processing of pre-mRNA takes place; and (4) Binding of DDX1 to the RRE could have a direct action, or act indirectly mediated by hnRNPK. Importantly, the region of DDX1 where hnRNPK binds overlaps the Rev-interacting region, which may affect the release of Rev from the RRE, with ATPase activity, in the cytoplasm.

By combining interpretations of this report and the above findings and known enzymatic activities of DDX1 (Chen et al., 2002), we propose a potential model for the effects of DDX1 on the HIV-1 life-cycle. DDX1, by maintaining spatial structure of Rev that binds to RRE, may act for an effective Rev-RRE binding in nucleus; by utilizing its RNA helicase and ATPase activities, DDX1 may act on altering RRE spatial structure with consumption of ATP for release of Rev bound to RRE in the cytoplasm. Regardless of presence of the RRE, Rev subcellular localization was altered in DDX1 down-regulated cells from nuclear to cytoplasmic dominance. We suggest that a decrease in interaction between NIS and DDX1 molecules may be responsible for relative sequestering of Rev in the cytoplasm, as a result of a dynamic imbalance of Rev trafficking through the nuclear pore complex (NPC), rather than an impaired nuclear/nucleolar localization process possibly mediated by importin-β and the putative Rev cofactor, B23 (Fankhauser et al., 1991; Kjems and Askjaer, 2000). Of note, intra-nuclear localization differences between Rev and DDX1 may inhibit DDX1-induced Rev release from the RRE, while both are in the nucleus of HIV-1-infected cells.

RNA helicases are known to be involved in virtually every aspect of RNA metabolism, including transcription, splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, and RNA decay (Bleoo et al., 2001; de la Cruz et al., 1999; Linder and Stutz, 2001; Tanner and Linder, 2001). Nevertheless, the biological functions of DDX1 are not fully understood. Pathologically, DDX1 is found amplified in certain neuroblastoma and retinoblastoma cell-lines (Godbout et al., 1998), and thus it may play certain roles in the oncogenesis of neural tissues.

Restriction of HIV-1 replication occurs in a variety of host cells, which could be due to the action of cellular factors, through either over-expression of inhibitory factors and/or lack of factors required by HIV-1. For example, CEM15 or APOBEC-3G is an endogenous inhibitor of HIV-

1 expressed in cell-types in which the absence of HIV-1 Vif yields a "non-permissive" phenotype for production of viable virions and can cause hypermutation in newly synthesized retroviral DNA (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Sheehy et al., 2002; Zhang et al., 2003).

Interestingly, the inter-relationship between down-regulated DDX1 and restricted HIV-1 replication is reminiscent of that found in human astrocytes and certain glial cell-lines. In those cells, wild-type Rev is not functioning properly due to a cellular background which induces aberrant subcellular distribution of Rev [(Neumann et al., 1995, 2001) and our unpublished studies]. In primary astrocytes as well as a glioma cell-line, transiently expressed Rev was localized dominantly in the cytoplasm, which is strikingly similar to our findings in the DDX1-down-regulated Cos-1 cells. Such unfavorable microenvironments for Rev contribute to the inefficiency of HIV-1 replication, as supported by a report describing Rev inhibition altering intracellular HIV-1 RNA distribution (Cmarko et al., 2002). Indeed, this similarity may be a critical clue leading to the elucidation of mechanisms involved in restricted HIV-1 replication in human astrocytes and other cell-types.

Finally, if one can reduce the DDX1 expression level to the least required physiological concentrations in HIV-1 susceptible cells, viral persistence or latency may develop upon HIV-1 infection due to reduced Rev functions (Pomerantz et al., 1990). As such, these findings demonstrate both a new class of cellular cofactors involved in productive HIV-1 infection and reveal a potential new set of molecular targets for anti-HIV-1 therapeutics.

Materials and methods

Plasmids, siRNAs, and PCR

pcRev expresses wild-type Rev and was reported previously (Malim et al., 1989). pcDDX1 was derived from pBluscript SK-DDX1 (Godbout and Squire, 1993), by inserting the 2.7 kb *Bam*H1–*Xho*I DDX1 gene into pcDNA 3.0. pSG_5Gal-VP, pSG_5Gal-Rev and pSG_5Gal-Revd23 were described previously (Fang et al., 2002). pSG_5Gal-NIS was constructed by inserting a NIS-encoding oligonucleotide into *Eco*R1–*Bam*I sites of pSG_5Gal-VP. pSG_5NISYP-1-VP was constructed by inserting polymerase chain reaction (PCR)-generated NISYP-1 into EcoR1–HindIII sites of pSG_5Gal-VP. The HIV-1 Rev and DDX1 genes, generated by PCR, were inserted in pCITE-4 (Novagen, Madison, WI), a vector for in vitro translation. The Rev and DDX1 genes were also inserted into the pGEX vector for in vitro expression and isolation of GST-Rev and GST–DDX1 fusion proteins. The initial plasmids for the in vivo RNA binding assay, pcTat/Rev, and pSLIIBCAT, were described previously (Madore et al., 1994). pSLIIBLuc was constructed by replacing the chloramphenicol acetyl transferase (CAT) gene between *Hin*dIII and *Bam*H1 in pSLIIBCAT with the luciferase gene. pcTat/DDX1 was constructed by replacing Rev in pcTat/Rev with *Xho*I–*Nco*I full-length DDX1. pDM128 (Hope et al., 1990), a reporter vector for Rev function, was modified by replacing the *Not*I–*Bam*H1 CAT gene in pDM128 with a PCR-generated Not I–Bgl II luciferase gene. We entitled the resultant vector, pDM628. The target site for the siRNA DDX1 duplex was: CAAGCCCTCTTTCCTGCCTG (961–980, X70649; Dharmacon, Lafayette, CO). The vector for expressing antisense RNA to DDX1 (pAs-DDX1) was constructed from pcDNA3.0, by deleting *Nru*I–*Sna*BI of the cytomegalovirus (CMV) promoter region and inserting the human U6 promoter (X07425), followed by an oligonucleotide encoding the DDX1 target site, as above, with poly (T). pCMV-HA-DDX1 was constructed by inserting PCR-generated DDX1 (*Sal*I–*Bam*H1) into the *Sal*I–*Bgl*II sites of pCMV-HA (BD Clontech). All constructs were confirmed via sequence analyses. Three pairs of reverse transcriptase polymerase chain reaction (RT-PCR) primers, SC05F/628RTS, SC05F/628RTU and hGAPDH-F/hGAPDH-R were used for detection of spliced pDM628 (240 bp), unspliced pDM628 (300 bp) and intron-spanning hGAPDH (540 bp), respectively. The primer sequences were: SC05F 5′GAAGAAGCGGAGACAGCGACGAAGAGCTC3′, 628RTS 5′ CTAACAGCTGCCTTGTAAGTCATTGGTC-T3′, 628RTU 5′CCAGCGGTTCCATCCTCTAGAGGA-TAGA3′, hGAPDH-F 5′CCATCACCATCTTCCAGG-AGCGAGATC3′, hGAPDH-R 5′CAGGTTTTTCTAG-CGGCAGGTCAGG3′. Cell fractionation, RNA extraction techniques, and RT-PCR parameters followed the manufacturer's protocol (PARIS and Retroscript Kits, Ambion, Austin, TX), with minor modifications.

Yeast and mammalian two-hybrid systems

The yeast two-hybrid system was purchased from Clontech (MATCHMAKER LexA two-hybrid system; Cat. K1609-1). A cDNA library derived from the Jurkat T-cell-line (Clontech #HL4513AK) served as the source in searching for NIS cellular co-factors. The screening process followed the manufacturer's instructions with modifications. A Gal4-VP16-based mammalian two-hybrid system was employed to examine protein–protein interactions between DDX1 and Rev molecules in vivo, with modifications of the reporter construct, using the β-Gal gene instead of the CAT gene (see Fig. 1 legend; Bogerd and Greene, 1993; Shimano et al., 1998).

Protein expression and GST pull-down assays

Expression of GST, GST–Rev and GST–DDX1 fusion proteins, in vitro translation of Rev and DDX1, and GST pull-down assays were carried out by following protocols, as described previously (Yang et al., 2001). Briefly, GST, GST–Rev and GST–DDX1 proteins were expressed in

competent BL21 cells that were transformed with pGEX, pGEX–Rev and pGEX–DDX1 vectors, respectively, and then induced with isopropylthio-β-D-galactoside. The induced cells were lysed and applied to glutathione-conjugated agarose bead columns. After intensive washes, the beads were aliquoted and stored at −20 °C. ^{35}S-labeled Rev and DDX1 were synthesized utilizing SPT3 kits (Novagen), following the manufacturer's protocol. For GST pull-down assays, GST, GST-DDX1, and GST-Rev-conjugated bead slurries were mixed with ^{35}S-labeled Rev or DDX1 in a binding buffer (150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 0.1% Triton X-100). After binding at 4 °C for 1 h, the mixtures were centrifuged, and the beads were washed with binding buffer three times. The ^{35}S-Rev and DDX1 proteins were dissociated from beads by adding SDS-containing loading buffer, and heating at 95 °C for 5 min. The samples were then electrophoresed on 4–20% SDS-PAGE gels, and the gels were dried and exposed to X-ray film.

Cell cultures, transfections, and assays

Cos-1 and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in humidified air containing 5% CO_2 at 37 °C. HLfb *rev*(−) cells were kindly provided by the AIDS Reagent Program (N.I.H.; Mermer et al., 1990). Plasmid and siRNA transfections were carried out following the manufacturer's optimized protocols (Fugen6: Roche). Transfected cells were recovered 48 h post-transfection for reporter and protein expression assays, with the exception of the 24 h post-transfection samples for luciferase activity assays (BD PharMingen, San Diego, CA), using a FB12 luminometer (Berthold Detection System USA, Oak Ridge, TN). Rev-RRE interactions in the cells were evaluated by a system to monitor the RRE-mediated *trans*-activation of the luciferase gene by the Tat–Rev protein fusions, as originally described using CAT as a reporter gene (Madore et al., 1994). Beta-galactosidase (β-Gal) assays were carried out 48 h after transfection, by following maker's protocol (BD Clontech, Palo Alto, CA). The optical absorbency at a wavelength of 420 nm of each sample was computed in β-Gal units, according to a standard curve obtained with sample standards.

Antibodies, immunofluorescence analyses, Western blotting, and co-immunoprecipitation assays

The rabbit antiserum, Rev 1/20, and rabbit anti-DDX1 antiserum (Godbout et al., 1998) were utilized in the detection of HIV-1 Rev and cellular DDX1, respectively, at dilutions of 1:200 in immunofluorescence analyses (IFA). The fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Sigma) was employed as a secondary antibody. Mouse anti-DDX1 monoclonal antibody (BD PharMingen) and anti-Gal4 murine monoclonal antibody were used in co-immunoprecipitation studies (BD Clontech, Palo Alto, CA). The co-immunoprecipitation were carried out according the protocols described previously (Yang et al., 2001). In brief: HEK293 cells were transfected with pcDDX1 and pcRev or pGal4Rev. The lysates were treated with rabbit-anti-Rev (1:1000), rabbit-anti-DDX1 (1:1000) polyclonal antibody, and normal rabbit serum as a negative control, respectively, followed by adding protein A beads (Amersham) with another hour binding reaction, followed by washing with NETN buffer. The beads were then electrophoresed on a 4–20% SDS-PAGE gel. Western blotting was utilized to detect specific DDX1 and Rev proteins, by using monoclonal mouse-anti-DDX1 antibody and monoclonal mouse-anti-Gal4 antibody, respectively, as primary antibodies, and HRP-conjugated anti-mouse as a secondary antibody, the specific DDX1 and Rev bands were detected, respectively, utilizing an ECL kit (Amersham).

For Western blotting of DDX1, anti-HA-peroxidase antibody (Cat. #2013819, Roche) was used to detect HA-DDX1. Mouse anti-human β-actin was purchased from ABCam (ab6276, ABcam, Inc., Cambridge, MA).

Acknowledgments

The authors thank the AIDS Reagent Program, Division of AIDS, NIAID, NIH, for providing the HLfb *rev*(−) line that was donated by Drs. Barabara Felber and George Pavlakis; Dr. Tristram G. Parslow for the pDM128 construct, Dr. Michael H. Malim for pcRev, Dr. Bryan R. Cullen for providing a rabbit anti-Rev antibody, and Dr. Keyang Chen and Ms. Chune Zhang for helpful suggestions and technical assistance. We also thank Ms. Rita M. Victor and Ms. Brenda O. Gordon for excellent secretarial assistance. This work was supported in part by US PHS grants AI43876, AI43289, NS41864 and NS27405 to R.J.P.

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