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Direct Participation of Sam68, the 68-Kilodalton Src-Associated Protein in Mitosis, in the CRM1-Mediated Rev Nuclear Export Pathway

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Human immunodeficiency virus type 1 (HIV-1) replication requires efficient nuclear export of incompletely spliced and unspliced HIV-1 mRNA transcripts, which is achieved by Rev expression at an early stage of the viral life cycle. We have recently shown that expression of Sam68, the 68-kDa Src-associated protein in mitosis, is able to alleviate Rev function block in astrocytes by promoting Rev nuclear export. In the present study, we utilized an antisense RNA expression strategy to down-modulate constitutive Sam68 expression and examined its effect on Rev function, HIV-1 gene expression, and viral replication. These results showed that down-modulation of constitutive Sam68 expression markedly inhibited HIV-1 production in 293T cells and viral replication in T lymphocytes such as Jurkat and CEM cells, as well as human peripheral blood mononuclear cells (PBMCs). Rev-dependent in *trans* complementation and reporter gene assays further demonstrated that inhibition of HIV-1 gene expression by Sam68 down-modulation was due to impeded Rev activity. Moreover, digital fluorescence microscopic imaging revealed that down-modulation of Sam68 expression caused exclusive nuclear retention and colocalization of both Rev and CRM1. Taken together, these data suggest that adequate Sam68 expression is required for Rev function and, thereby, for HIV-1 gene expression and viral replication, and they support the notion that Sam68 is directly involved in the CRM1-mediated Rev nuclear export pathway.

Transport of macromolecules such as proteins and RNAs between the nucleus and the cytoplasm via the nuclear pore complex (NPC) is a highly regulated process, which involves multiple interactions between import or export macromolecules, cellular factors, and components of the NPC (for reviews, see references 51, 65, and 72). In higher eukaryotic cells, only completely spliced mRNA transcripts are able to be transported by the normal mRNA export pathway into the cytoplasm for translation, while intron-containing mRNAs are confined to the nucleus (78). Human immunodeficiency virus type 1 (HIV-1) encodes three classes of mRNA transcripts: completely spliced (~2-kb), incompletely spliced (~4-kb), and unspliced (~9-kb) mRNAs (21, 46, 61, 67). In order to export the incompletely spliced and unspliced RNAs into the cytoplasm for synthesis of viral structural components and the viral genome, HIV-1 has evolved not only to generate the ~4- and ~9-kb mRNAs by inefficient and alternative splicing (48, 57, 67) but also to transport these two intron-containing mRNAs from the nucleus to the cytoplasm by expression of Rev protein (40), which itself is translated from completely spliced ~2-kb mRNAs. Hence, Rev function divides the HIV-1 life cycle into two phases: a Rev-independent early stage and a Rev-dependent late stage (12, 33).

Rev is a small nuclear and/or nucleolar protein (10, 40) that binds specifically to a highly structured RNA sequence called the Rev-responsive element (RRE), which is present in all incompletely spliced and unspliced mRNAs (22, 41, 63). Two distinct domains of Rev have been demonstrated to be essential for its functions: an arginine-rich N-terminal domain that controls three critical functions of Rev—binding to the RRE of incompletely spliced and unspliced mRNAs (27, 40), nuclear and nucleolar localization (15, 27), and Rev oligomerization (28, 77)—and a C-terminal domain, which contains a stretch of leucine-rich amino acid residues and functions as a nuclear export signal (NES) (15, 40). Although Rev is mainly expressed in the nucleus and/or nucleolus, it is evident that Rev constantly shuttles between the nucleus and the cytoplasm (30, 45, 59). Productive HIV-1 replication requires efficient Rev nucleocytoplasmic shuttling activity (14, 47, 74).

Several cellular factors have been identified to be directly involved in the Rev-mediated nuclear export pathway, such as CRM1 (16, 19), eukaryotic initiation factor 5A (eIF-5A) (64), and Rip/Rab (7, 18). CRM1, also known as exportin 1, has been well documented as a receptor for the leucine-rich NES-containing proteins (16). CRM1 binds to the leucine-rich NES-containing protein at the NES in the presence of the GTP-bound form of Ran and transports these proteins from the nucleus through the nuclear pore (2, 19, 49, 53). eIF-5A was initially identified as a NES-binding protein by UV cross-linking (64). Several mutants of eIF-5A have been shown to block the nuclear export of Rev protein and HIV-1 replication (6). Microinjection studies have further shown that antibodies directed against eIF-5A specifically abolish the nucleocytoplas-

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mic translocation of HIV-1 Rev in somatic cells (66), indicating that eIF-5A is part of a specific nucleocytoplasmic export pathway. In contrast, hRIP/Rab was cloned as a NES-interacting protein by using the yeast two-hybrid cloning technique (7, 18, 69). Recent studies have shown that these repeated phenylalanine-glycine (FG)-containing nucleoporins bind to the Rev NES indirectly via CRM1 (49).

Sam68 was initially isolated as the 68-kDa Src-associated protein in mitosis (20, 76) and has recently been shown to be able to substitute for and synergize with Rev function (58). A dominant-negative mutant, Sam68ΔC, in which the C-terminal nuclear localization signal is deleted has also been demonstrated to inhibit Rev nuclear localization and consequently Rev function and HIV-1 replication (58). During our recent attempts to understand the molecular mechanisms underlying abnormal Rev function in astrocytes, we found that expression of exogenous Sam68 is not able to substitute for Rev but is able to alleviate a Rev function block in cells expressing a lower level of constitutive Sam68 protein, such as astrocytes (35). We have further demonstrated that Sam68 expression results in Rev nuclear export by direct interaction (35). Moreover, a recent study has shown that Sam68ΔC-mediated inhibition of Rev activity is due to perinuclear sequestering of unspliced HIV-1 mRNAs (68). These discrepancies led us to further characterize the roles of Sam68 protein in Rev function and HIV-1 replication by using an antisense RNA expression vector-based strategy. Our results demonstrate that down-modulation of Sam68 expression markedly inhibited HIV-1 gene expression, replication, and Rev function and that the inhibition was due to a block of CRM1-mediated Rev nuclear export. These data together suggest that Sam68 plays a direct role in CRM1-mediated Rev export from the nucleus to the cytoplasm.

MATERIALS AND METHODS

Cell cultures and transfections. 293T, Jurkat, CEM, and BOSC23 cells were purchased from the American Tissue Culture Collection and maintained in either Dulbecco's modified Eagle's medium (293T and BOSC23 cells) or RPMI 1640 medium (Jurkat and CEM cells) supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll-Hypaque, as described previously (55), and cultured in complete RPMI 1640 medium containing 10% fetal calf serum. Cell transfections were performed by the calcium phosphate precipitation method, which usually gives rise to 80% or higher transfection efficiency in 293T and BOSC23 cells. pcDNA3 was used to equalize the amount of DNA transfected among all transfections throughout the studies. pCMVβGal was included to normalize variations of transfection efficiency among all transfections involving the chloramphenicol acetyltransferase (CAT) reporter gene assay, as previously described (23, 24).

Plasmids. The sources of plasmids used in the studies were as follows: HIVΔenv and HIVΔenvΔrev were from M. Emerman (34), pSam68.HA from T. Ishidate (29), pSG5.Sam68 from S. Courtneidge (37), pGFP.Rev from R. Brack-Werner (38), pc.Rev and M10 from B. Cullen (40), pMX and pMX-IL-3R from T. Kitamura (52), pRRE-CAT and pPRE-CAT from T. Hope (27), and pSV2-CAT and pCTE-CAT from J. Roth (60). pcDNA3, pDsRed1-C1, and pEGFP-C1 were purchased from Clontech. pSam68.RFP and pRev.RFP have been described previously (35). The standard PCR cloning technique was used to construct the following expression plasmids (with the templates, primers, and cloning backbones shown in parentheses and the restriction enzyme sites underlined): pAs.Sam68a (pSG5.Sam68, 5′-TAA TGA ATT CAT GCA GCG CCG GGA CGA C-3′ and 5′-GGA ATT CTT AAT AAC GTC CAT ATG GGT G-3′, pcDNA3), pCRM1.RFP (pc3.CRM1, 5′-CGG GGT ACC ATG CCA GCA ATT ATG ACA ATG-3′ and 5′-CGC GGA TCC TTA ATC ACA CAT TTC TTC TGG-3′, pDsRed1-C1), and pCRM1.GFP (same template and primers as for pCRM1.RFP, with pEGFP-C1 as the cloning backbone). pAs.Sam68b was constructed by cloning the entire Sam68 insert (~1.7 kb) from pSG5.Sam68 by *Eco*RI digestion, followed by ligation of the insert into pcDNA3 at the *Eco*RI site in a reverse orientation. pMX.As-Sam68 was constructed in a similar manner, except that a different backbone, i.e., pMX, was used. All recombinant plasmids were verified by sequencing, and functional activities of pRev.GFP and pM10.GFP were confirmed by both an RRE-mediated reporter gene assay and an in *trans* complementation assay.

Preparation of whole-cell lysates and Western blot analysis. Cells were washed twice with ice-cold phosphate-buffered saline and then harvested with a cell harvester. Cell pellets were resuspended in 2 volumes of whole-cell lysis buffer (10 mM $NaHPO_4$, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.2% sodium azide, 0.5% sodium deoxycholate, 0.004% sodium fluoride, 1 mM sodium orthovanadate) and incubated on ice for 10 min. Whole-cell lysates were obtained by centrifugation and removal of cell debris. Cell lysates (25 μg of protein) were electrophoretically separated on sodium dodecyl sulfate–10% polyacrylamide gels and analyzed by immunoblotting using antibodies against Sam68 (Santa Cruz). The blots were then probed with appropriate horseradish peroxidase-conjugated secondary antibodies and visualized with the ECL system (Amersham). Relative levels of protein expression were determined by densitometric scanning of the blots using the housekeeping gene actin as an internal standard.

Virus stocks and RTase assay. NL4-3 virus stocks were made by transfection of 293T cells with HIV-1 NL4-3 proviral DNA as previously described (1). Cell culture supernatants were collected 48 h after medium change, filtered, and saved as NL4-3 virus stocks. Replication-competent xenotropic murine leukemia virus (MLV) BV2 stocks were prepared as described previously (39). pMX-based viruses were prepared in a similar manner, except that a different cell line, i.e., the packaging cell line BOSC23, was used, as described elsewhere (56). For infection, cells were cultured on 10-cm-diameter plates and infected with viruses as indicated, in the presence of 8 μg of Polybrene/ml at 37°C for 2 h. Cells were then washed and supplemented with fresh medium. Viral infection and replication were determined by measuring reverse transcriptase (RTase) activity as described elsewhere (24). To assay the RTase activity of the BV2 or pMX virus, we adopted a similar protocol, except that magnesium was replaced with manganese in the RTase assay buffer.

Digital fluorescence microscopic imaging analysis. Fluorescence images were captured by using a digital video imaging microscope system consisting of an Axiovert 200 M microscope with a 40× 1.4 UVF objective and an Axiovision video camera (all from Carl Zeiss) and were analyzed on an IDE Imaging Workstation (Tegraf).

RESULTS

Inhibition of HIV-1 production in 293T cells by down-modulation of Sam68 expression. To determine the functional significance of Sam68 protein in HIV-1 gene expression, we down-modulated constitutive Sam68 expression and investigated its effect on HIV-1 production. We took advantage of an antisense RNA expression vector-based strategy which has been widely and successfully used to study the functions of a number of genes (for a review, see reference 73). We made two Sam68 antisense constructs that expressed different lengths of the Sam68 cDNA in a reverse orientation in the context of a pcDNA3 backbone. 293T cells were transfected with these two antisense RNA expression plasmid DNAs. Western blot analysis showed that transient expression of these two anti-Sam68 antisense RNAs effectively down-regulated Sam68 protein expression by 67 to 69% (Fig. 1a). To determine whether there was any adverse effect on these cells transfected with anti-Sam68 antisense RNAs, we performed [^{3}H]thymidine uptake, cell cycle, and cell viability analyses. However, no changes in cell proliferation, cell cycling time, or cell survival were detected in these transfected cells during the experiments, i.e., 2 to 7 days (data not shown).

We then transfected 293T cells with HIV-1 NL4-3 proviral DNA, in combination with or without anti-Sam68 antisense RNA expression vectors, and monitored viral production in

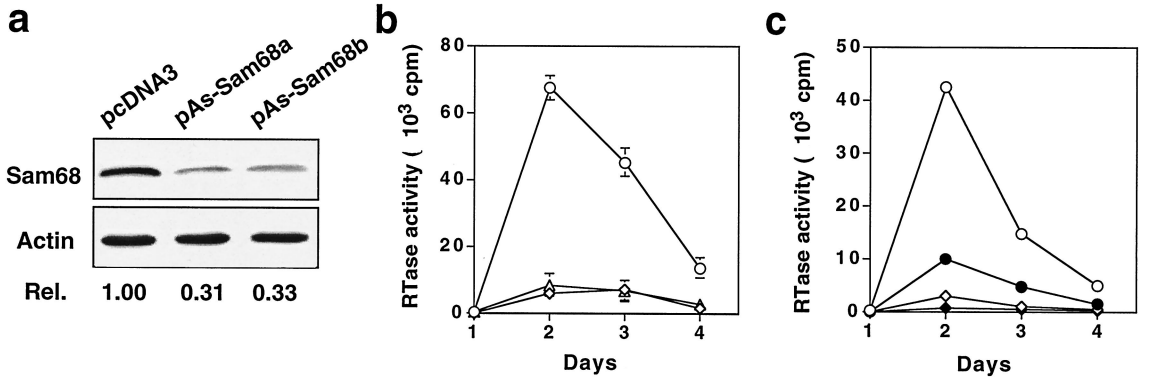

FIG. 1. Inhibition of HIV-1 production by down-modulation Sam68 expression. (a) 293T cells were transfected with one of two anti-Sam68 antisense RNA constructs (6 μg), one containing a longer Sam68 cDNA (1.7 kb) in a reverse orientation (pAs-Sam68a) and the other containing only the 1.3-kb open reading frame of Sam68 cDNA in a reverse orientation (pAs-Sam68b), and were harvested 48 h after transfection for Sam68 expression. Sam68 expression was analyzed by Western blotting. pcDNA3 was also included as a transfection control. Rel., relative levels of protein expression determined by densitometric scanning of the blots using the housekeeping gene actin as an internal standard. (b) 293T cells were transfected with 1.5 μg of HIV-1 NL4-3 proviral DNA with 6 μg of either pcDNA3 (○), pAs-Sam68a (△), or pAs-Sam68b DNA (◇). HIV-1 production was determined by measuring the RTase activity of the cell culture supernatant at the time points indicated. (c) 293T cells were transfected with 1.5 μg of HIV-1 NL4-3 proviral DNA with 0 (○), 2 (●), 4 (◇), or 6 (◆) μg of pAs-Sam68a DNA. pcDNA3 was used to equalize the total amount of DNA transfected among all transfections. HIV-1 production was determined as described above.

these cells. The results showed that expression of either anti-Sam68 antisense RNA construct inhibited HIV-1 production by 80 to 86% (Fig. 1b). Inhibition of viral production was correlated with the amount of Sam68 antisense RNA expression vector DNA transfected (Fig. 1c). To ascertain that the inhibitory effect of Sam68 down-modulation on HIV-1 production is not due to a defect in virus budding in cells transfected with an anti-Sam68 antisense RNA expression vector, we also determined intracellular virus production levels in transfected 293T cells. The results showed a similar inhibitory effect (data not shown), further suggesting that transient HIV-1 gene expression and virus production were inhibited by down-modulation of constitutive Sam68 expression.

Inhibition of HIV-1 replication by down-modulation of Sam68 expression. We then determined whether down-modulation of constitutive Sam68 expression would also affect HIV-1 replication in human T lymphocytes and PBMCs. To achieve a higher level of anti-Sam68 antisense RNA expression, we utilized a retrovirus-based gene delivery system, pMX, which has been shown to be very efficient at introducing foreign genes into a variety of highly refractory cell lines and primary cells (52). We cloned the 1.7-kb Sam68 cDNA in a reverse orientation into the retroviral pMX vector (pMX.As-Sam68) and prepared the viruses using the BOSC23 packing cell line (56). We transduced human T lymphocytes, Jurkat and CEM cells, and human PBMCs with the MX.As-Sam68 viruses expressing anti-Sam68 antisense RNA. The transduction efficiency was determined to be about 26% by using pMX-IL-3R as the control for interleukin-3 receptor expression (52). We then infected the transduced cells with HIV-1 NL4-3 and monitored the cells for HIV-1 replication. During the first 6 days postinfection, cells transduced with MX control viruses containing no Sam68 insert exhibited a kinetics typical of productive HIV-1 replication (Fig. 2a). In contrast, cells transduced with MX.As-Sam68 viruses produced little or no virus (Fig. 2a). It has been documented that down-modulation of constitutive gene expression by the antisense RNA technology is a result of double-stranded RNA formation between mRNA and complementary antisense RNA, so that mRNA is not accessible for translation (for a review, see reference 73). On the other hand, it has also been known that formation of double-stranded RNAs may give rise to a nonspecific inhibitory effect on viral replication (13, 62). Thus, we included as an infection control a recombinant replication-competent, Rev-independent simple retrovirus, MLV strain BV2 (39), in order to determine whether double-stranded RNA formation between Sam68 mRNA and anti-Sam68 antisense RNA resulted in a nonspecific antiviral effect. No differences were noted in MLV BV2 viral replication between cells transduced with MX viruses and cells transduced with MX.As-Sam68 viruses (Fig. 2b), suggesting that a nonspecific antiviral effect of double-stranded RNAs is not responsible for the inhibition of HIV-1 replication by anti-Sam68 antisense RNA. In agreement with our previous findings that adequate Sam68 expression is required for productive HIV-1 gene expression (35), these results demonstrated that down-modulation of constitutive Sam68 expression inhibited HIV-1 replication in human T lymphocytes and PBMCs.

The apparent discrepancy between the transduction efficiency (26%) and the inhibition efficiency (98%) (Fig. 2a) raised a possibility that the inhibitory effect on HIV-1 gene expression resulting from down-modulation of constitutive Sam68 expression was not cell autonomous. To address this possibility, we transduced T lymphocytes (Jurkat cells) with the same amount of pMX.As-Sam68 virus expressing anti-Sam68 antisense RNA as that used for Fig. 2a, collected the cell culture supernatant 48 h after transduction, and determined its effects on HIV-1 replication in Jurkat cells that were infected with HIV-1 NL4-3. We also included pMX and pMX.GFP viruses as transduction controls. The transduction efficiency was 24.6% based on green fluorescent protein (GFP) expression in pMX.GFP virus-transduced cells, which was close to that previously obtained. The results showed that, like pMX viruses and mock transduction (conditioned medium), the su-

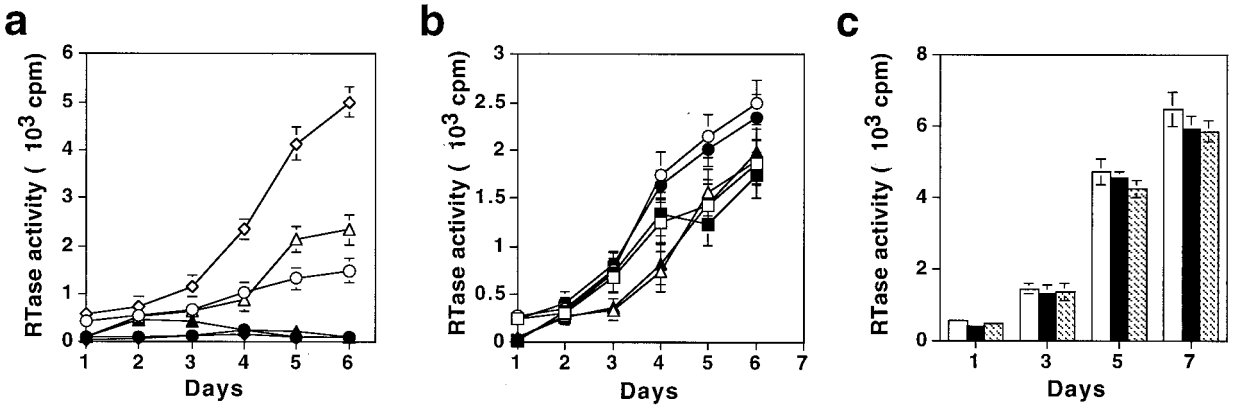

FIG. 2. Inhibition of HIV replication by down-modulation of Sam68 expression. (a and b) pMX.As-Sam68, containing the longer (1.7-kb) Sam68 cDNA in a reverse orientation, was transfected into BOS23 packaging cells by the standard calcium phosphate precipitation method (52), and the culture supernatant was collected 48 h after transfection as virus stocks. Jurkat cells (diamonds), CEM cells (circles), and human PBMCs (triangles) were first transduced with MX viruses (open symbols) or MX.As-Sam68 viruses (solid symbols) for 2 h, and then the free viruses were washed off with fresh cell culture medium. MX virus-transduced cells were immediately infected with HIV-1 NL4-3 with an RTase activity of 20,000 cpm (a) or with an equivalent amount of MLV BV2 (b) for an additional 2 h; then the free viruses were washed off with fresh cell culture medium again, and cells were allowed to grow at 37°C under 5% CO_2. (c) Inhibition of HIV-1 replication is cell autonomous. Jurkat cells were transduced either with pMX viruses (solid bars) or pMX.As-Sam68 viruses (hatched bars) or without MX viruses (mock transduction) (open bars) as described above. The cell culture supernatant was collected 48 h after transduction and added to Jurkat cells infected with HIV-1 NL4-3. HIV-1 replication was determined by measuring the RTase activity of the cell culture supernatant (0.5 ml) at the time points indicated.

pernatant from pMX.As-Sam68-transduced cells failed to exhibit any inhibitory effect on HIV-1 replication in Jurkat cells (Fig. 2c). We also tested supernatants collected at 0, 6, 12, 24, and 72 h after MX viral transduction and found no effects (data not shown). These results together suggest that inhibition of HIV-1 gene expression by down-modulation of Sam68 expression was cell autonomous. Furthermore, these results also validated the findings of earlier studies that infection with MLV or MLV-based retroviruses promotes or facilitates coinfection with HIV-1 (8, 9, 71).

Inhibition of Rev-dependent gene expression by down-modulation of Sam68 expression. Since our previous studies (35) and studies by others (58, 68) have shown that Sam68 interaction with Rev may modulate Rev activity, we then determined whether inhibition of HIV-1 gene expression and viral replication by down-modulation of constitutive Sam68 expression is due to a decrease in Rev activity. We first performed an in *trans* complementation assay in the context of the HIV-1 genome. We transfected 293T cells with HIV.Δ*env*Δ*rev*, containing both *env* and *rev* deletions (4), and with either an expression vector for Rev, an expression vector for anti-Sam68 antisense RNA, or both. We also included HIV.Δ*env* (34), containing an *env* deletion but an intact *rev* gene, as a control. As expected, deletion of the *rev* gene from the HIV-1 genome, i.e., HIV.Δ*env*Δ*rev*, resulted in a minimal level of HIV-1 production, which was fully compensated for in *trans* by expression of a Rev expression plasmid (Fig. 3). Anti-Sam68 antisense RNA expression decreased Rev-mediated HIV-1 production by 64 and 82%, which appeared to be correlated with the amount of anti-Sam68 antisense RNA expression vector DNA transfected (Fig. 3). However, increasing Rev expression did not abolish the inhibitory effect on HIV-1 production in cells transfected with a larger amount of anti-Sam68 antisense RNA expression vector DNA (Fig. 3). These results demonstrated that down-modulation of constitutive Sam68 expression inhibited Rev activity, further supporting the notion that Sam68 expression is required for Rev function (35).

To determine the inhibitory specificity of Sam68 down-modulation on Rev function, we expressed several known Rev-independent reporter DNAs in the presence of an anti-Sam68 antisense RNA expression vector. These included a Mason-Pfizer monkey virus RNA constitutive transport element (CTE)-dependent CAT reporter (CTE-CAT) (60), a hepatitis B virus posttranslational regulatory element (PRE)-dependent CAT reporter (PRE-CAT) (54), and the simian virus 40 promoter-driven SV2-CAT reporter (60). We also transfected the Rev-dependent reporter RRE-CAT (54) as a control. As expected, Rev expression transactivated RRE-CAT expression

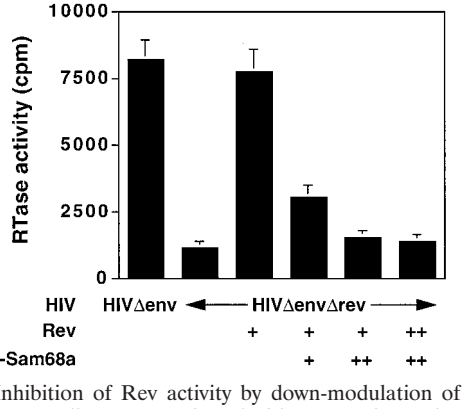

FIG. 3. Inhibition of Rev activity by down-modulation of Sam68 expression. 293T cells were transfected with 0.8 μg of HIV.Δ*env* alone or HIV.Δ*env*Δ*rev* alone, or with different expression plasmids as indicated. The amounts of DNA transfected were as follows: 0.2 μg (+) or 0.4 μg (++) of Rev; 3 μg (+) or 6 μg (++) of As-Sam68a. pcDNA3 was used to equalize the total amount of DNA transfected among all transfections. HIV-1 production was monitored by measurement of RTase activity.

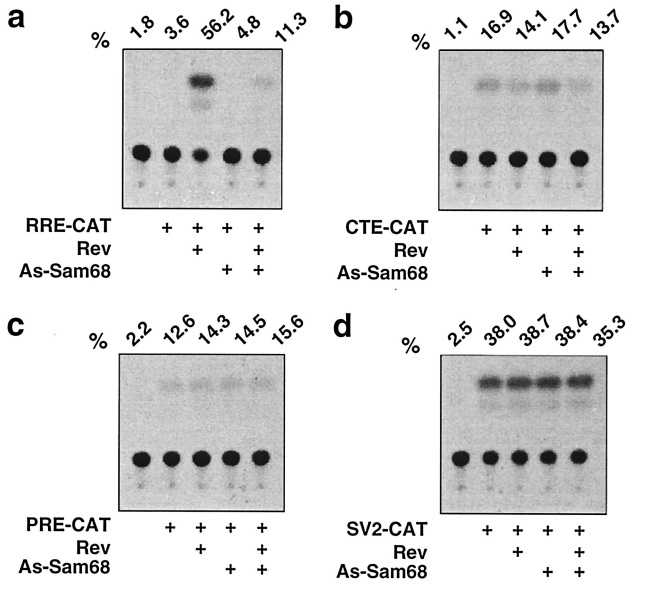

FIG. 4. Preferential inhibitory effect of Sam68 down-regulation on Rev activity. 293T cells were transfected with 0.5 μg of either RRE-CAT (a), CTE-CAT (b), PRE-CAT (c), or SV2-CAT (d), either alone or in combination with 0.2 μg of Rev and/or 3 μg of As-Sam68a. pcDNA3 was used to equalize the total amount of DNA transfected among all transfections. pCMVβGal was included to normalize variations in transfection efficiency among all transfections. Cells were harvested 48 h after transfection and assayed for CAT activity as previously described (23, 24). CAT activity was determined to be in the linear range.

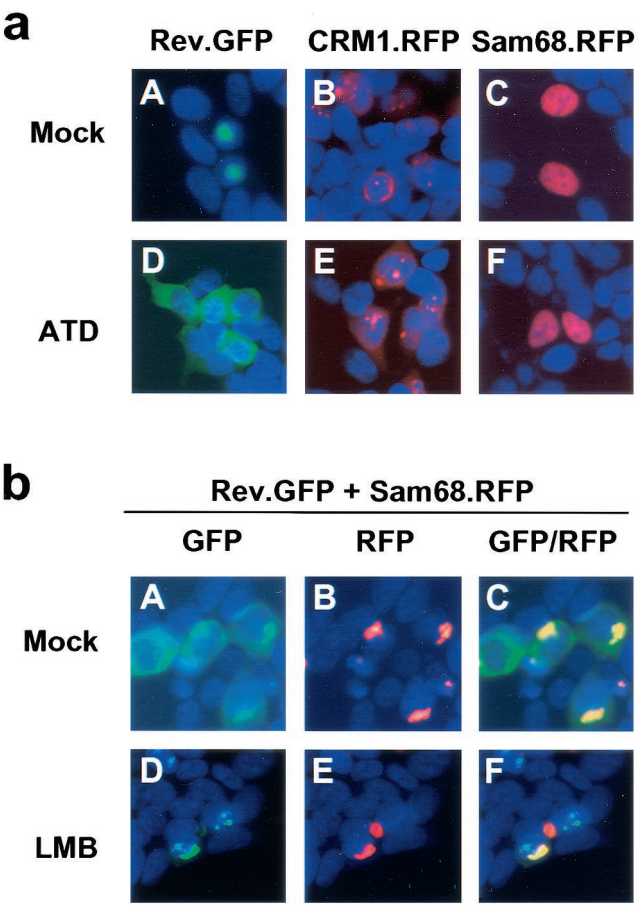

FIG. 5. Inhibition of Sam68 expression-induced Rev nuclear export by LMB treatment. 293T cells were transfected with either Rev.GFP, CRM1.RFP, or Sam68.RFP (a) or with both Rev.GFP and Sam68.RFP (b). Transfected cells were first treated with 2 μg of ATD/ml (a) or 10 ng of LMB/ml (b) 48 h after transfection, as indicated, and then fixed and stained with 1 ng of DAPI/ml for the nucleus. Image capturing and analysis were performed as described elsewhere (35).

by 54.4% (Fig. 4a) but exhibited little or no effect on CTE-, PRE-, or SV2-CAT expression (Fig. 4b through d). The results also showed that coexpression of anti-Sam68 antisense RNA resulted in a 44.9% decrease in Rev-mediated RRE-CAT expression (Fig. 4a) but in little or no inhibition of CTE-, PRE-, or SV2-CAT expression, regardless of whether Rev was expressed or not (Fig. 4b through d). These results together demonstrated that inhibition of Rev-dependent HIV-1 gene expression by Sam68 down-modulation was due to a preferential inhibition of Rev function.

Sam68 expression-induced Rev nuclear export is CRM1 dependent. Extensive studies have shown that Rev nuclear export is mediated by CRM1 (2, 16, 19, 32, 49, 53). Our previous studies have demonstrated that Sam68 expression promotes Rev nuclear export (35), suggesting that Sam68 may be also involved in the Rev nuclear export pathway. Thus, we decided to determine the relationship between Sam68 expression-induced Rev nuclear export and CRM1-mediated Rev nuclear export. Although our previous studies have shown that Sam68 is expressed only in the nucleus (35), we first wanted to ascertain whether Sam68 protein itself can be shuttled between the cytoplasm and the nucleus. We transfected 293T cells with the Sam68.RFP expression plasmid, in which Sam68 was tagged with red fluorescent protein (RFP), and then treated the cells with actinomycin D (ATD), a transcriptional inhibitor that is known to be capable of inducing accumulation of nucleocytoplasmic shuttling proteins in the cytoplasm (30, 59, 60). We also included as controls in the transfection the Rev.GFP and CRM1.RFP expression plasmids, which were tagged with GFP and RFP, respectively. We also fixed the cells in 2% paraformaldehyde and stained the cells with 4′,6′-diamidino-2-phenylindole (DAPI) (blue fluorescence) to locate the nucleus. As expected, Rev was localized in the nucleus (Fig. A), while CRM1 was mainly localized at the nuclear membrane, with some in the nucleus and in the cytoplasm (Fig. B). When the transfected cells were treated with ATD, both Rev and CRM1 were considerably shifted into the cytoplasm (Fig. D and E). However, ATD treatment resulted in little or no cytoplasmic localization of Sam68 (Fig. F), which remained within the nucleus (Fig. C). Taken together with our previous finding that Sam68 alone was not able to substitute for Rev in the Rev-responsive element (RRE)-mediated gene expression (35), these results demonstrated that Sam68 itself is not a nucleocytoplasmic shuttling protein.

We then took advantage of an CRM1-specific inhibitor, leptomycin B (LMB), which is known to prevent CRM1 from binding to Rev and thus to disrupt Rev nuclear export (2, 16, 19, 32, 50, 75), to determine whether LMB treatment would inhibit Sam68 expression-induced Rev nuclear export. We transfected 293T cells with both Rev.GFP and Sam68.RFP and then treated the cells with LMB. In agreement with our pre-

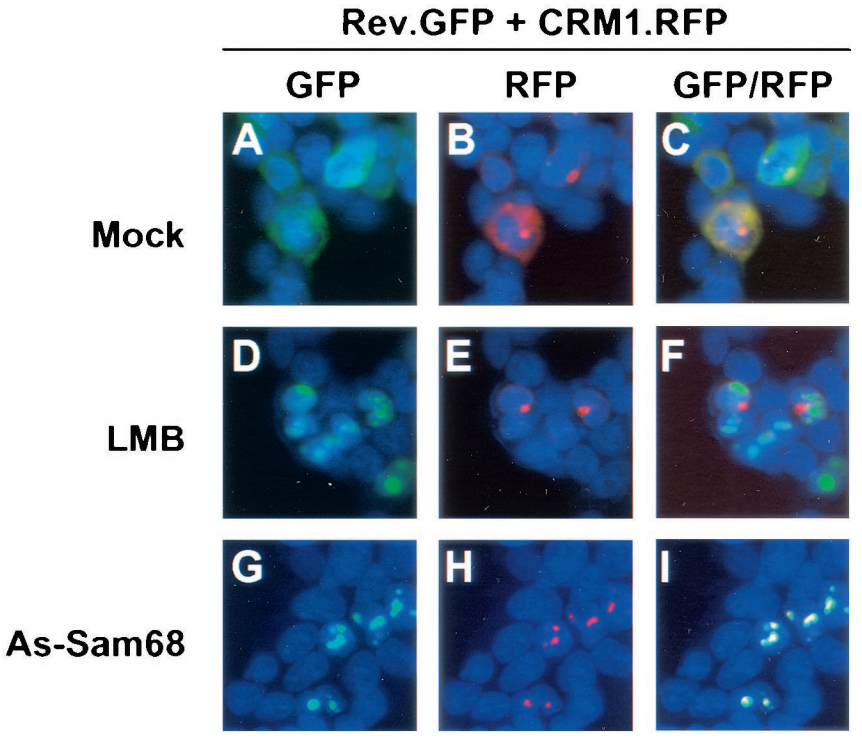

FIG. 6. Inhibition of CRM1-mediated Rev nuclear export by Sam68 down-modulation. 293T cells were transfected with the Rev.GFP and CRM1.RFP expression plasmids, either alone or in combination with the anti-Sam68 antisense RNA expression vector AS-Sam68a. pcDNA3 was used to equalize the total amount of DNA transfected among all transfections. LMB treatment, DAPI staining, and image capturing and analysis were performed as described above.

vious studies (35), the results showed that coexpression of Sam68 and Rev relocalized each from the nucleus (Fig. A and C) onto the nuclear membrane, which resulted in Rev nuclear export (Fig. A). Colocalization analysis showed that Sam68 was almost completely and exclusively colocalized with Rev on the nuclear membrane (Fig. C). Surprisingly, LMB treatment completely abolished Sam68 expression-induced Rev nuclear export, as Rev was retained in the nucleus (Fig. D) and Sam68 mainly remained on the nuclear membrane (Fig. E). Moreover, LMB treatment to some degree prevented colocalization of Sam68 and Rev (Fig. F) compared to that in the absence of LMB treatment (Fig. C). These results together demonstrated that Sam68 expression-induced Rev nuclear export was CRM1 dependent, suggesting that Sam68 may function as a previously unrecognized cofactor in the CRM1-mediated Rev nuclear export pathway.

Direct involvement of Sam68 in CRM1-mediated Rev nuclear export. To further characterize the roles of Sam68 in the CRM1-mediated Rev nuclear export pathway, we first determined whether CRM1 bound directly to Sam68. For this analysis we used immunoprecipitation combined with Western blotting of both constitutive proteins and transiently expressed proteins, as well as the yeast two-hybrid protein-protein interaction assay. Nevertheless, we were unable to detect any direct interaction between CRM1 and Sam68 in any of these experiments (data not shown). Although the NES of Rev has been demonstrated to be directly involved in the interaction of Rev with both Sam68 (35) and CRM1 (16, 19, 49), we did not detect any adverse effect of Sam68 expression on CRM1 binding to Rev, or vice versa (data not shown). These data suggest that Rev binding to Sam68 and to CRM1 may occur independently or simultaneously.

To determine the functional relationship between Sam68 and CRM1 in the Rev nuclear export pathway, we investigated the effect of Sam68 down-modulation on the CRM1-Rev interaction. We transfected 293T cells with Rev.GFP and CRM1.RFP in the absence or presence of the anti-Sam68 antisense RNA expression vector pAs-Sam68a (Fig. 1a). We also treated the transfected cells with LMB as a control. In agreement with the findings of previous studies (60, 79), the results showed that coexpression of CRM1 and Rev relocalized each to the nuclear membrane and led to Rev nuclear export (Fig. 6A and B). As expected, LMB treatment disrupted the CRM1-Rev interaction, resulting in no Rev nuclear export (Fig. 6D) and little colocalization of Rev and Sam68, as they were localized in distinct nuclear compartments (Fig. 6D and E). In contrast, down-modulation of Sam68 expression by expression of the anti-Sam68 antisense RNA expression vector As-Sam68a resulted in little or no Rev nuclear export (Fig. 6G) and exclusively nuclear localization of both Rev and CRM1 (Fig. 6G and H). Moreover, unlike LMB treatment (Fig. 6F), down-modulation of Sam68 expression also resulted in exclusive colocalization of Rev and Sam68 within the nucleus (Fig. 6I). Taken together, these results demonstrated that down-modulation of Sam68 expression blocked the CRM1-mediated Rev nuclear export pathway, suggesting that Sam68 expression-induced Rev nuclear export and CRM1-mediated Rev nuclear export were mutually dependent. In addition, these

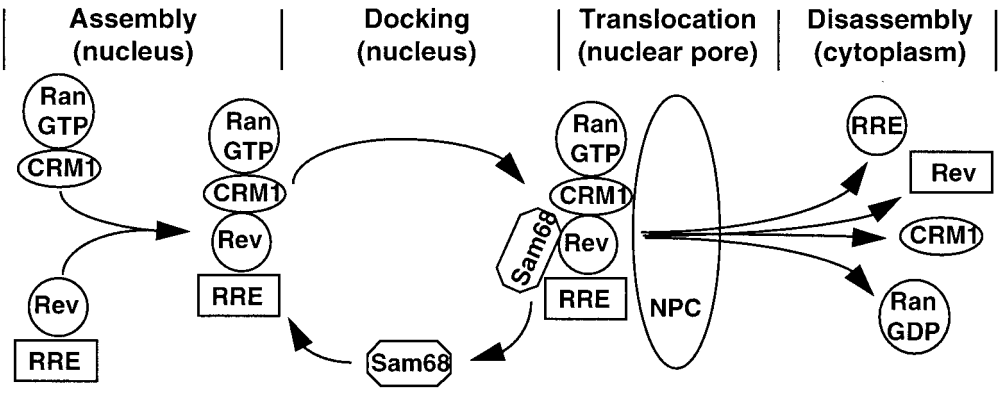

FIG. 7. Proposed model for Sam68 function in CRM1-mediated Rev nuclear export. Formation of a quadruple complex consisting of Rev, RRE-containing RNA, CRM1, and RanGTP occurs in the nucleus, possibly independently of Sam68. Sam68 then associates with the complex via direct binding to Rev and transports the complex to the NPC, followed by docking onto NPC through CRM1 interaction with nucleoporins. Translocation of Rev, CRM1, and RRE-containing RNAs into the cytoplasm leads to the release of Sam68 into the nucleus.

results also raised the possibility that Sam68 may serve to transport the export complex containing CRM1 and Rev from the nucleus to an area in close proximity to the nuclear membrane (the NPC).

DISCUSSION

By using the anti-Sam68 antisense RNA expression strategy, we have shown that down-modulation of Sam68 expression inhibited HIV-1 production in 293T cells and viral replication in human T-lymphoid cell lines such as Jurkat and CEM cells and human PBMCs. Our results from the in *trans* complementation assay and the Rev-dependent reporter gene assay showed that down-modulation of constitutive Sam68 expression preferentially inhibited the posttranscriptional transactivation activity of Rev protein. Sam68 has been shown to interact with Rev in a specific and direct manner (35, 58) and to promote Rev nuclear export (35). It has been well documented that Rev nuclear export is directly mediated by CRM1 (2, 16, 19, 32, 49, 53). Therefore, we decided to further elucidate the relationship between Sam68 expression-induced Rev nuclear export and CRM1-mediated Rev nuclear export. In agreement with results from other studies (44, 68), our results showed that Sam68 itself did not actively shuttle between the nucleus and the cytoplasm. In addition, our results showed that Sam68 expression-induced Rev nuclear export was CRM1 mediated and that down-modulation of Sam68 expression caused the complex containing CRM1 and Rev to be retained in the nucleus and thus blocked Rev nuclear export. Taken together, these data suggest that adequate Sam68 expression is required for Rev function by directly regulating the CRM1-mediated Rev nuclear export pathway.

Sam68 was first identified as a tyrosine-phosphorylated protein in Src-transformed NIH 3T3 cells (20, 76) and has been proposed to be involved in intracellular signal transduction and RNA metabolism (for a review, see reference 70). Previous studies by Reddy and colleagues have found that the dominant-negative mutant Sam68ΔC, in which the C-terminal nuclear localization signal has been deleted, impedes Rev nuclear localization and thus inhibits Rev function in Rev-dependent gene expression and HIV-1 replication (58). However, a recent study has disputed that finding and demonstrated that Sam68ΔC-induced inhibition is due to perinuclear sequestration of unspliced mRNAs, which prevents them from being translated (68). These discrepancies have yet to be investigated and resolved.

Sam68 has been demonstrated to preferentially bind to the RNA sequence UAAA (36). We located this sequence within stem I of the RRE, which corroborated the findings of previous studies that Sam68 specifically binds to the RRE (58). However, extensive studies have shown that only stems IIB and IID of the RRE are essential for Rev binding and thus for Rev-mediated nuclear export of viral RNAs (3, 5, 11, 25, 26, 31, 42). Our previous studies have shown that Sam68 expression alone is not able to substitute for Rev in activating Rev-dependent gene expression and HIV-1 replication (35). These data together suggest that Sam68 binding to the RRE may not be necessary for the Sam68 function in Rev nuclear export.

Sam68 has been demonstrated to bind to Rev (35, 58), and a double mutation (L78D E79L) within the NES of Rev disrupts the binding of Rev to Sam68 (35). The NES of Rev has also been found to interact with CRM1 (16, 19, 49). Thus, it is conceivable that Sam68 would compete with CRM1 for Rev binding. However, we were unable to detect any inhibitory effect of Sam68 expression on the binding of CRM1 to Rev or any inhibitory effect of CRM1 expression on the binding of Sam68 to Rev, suggesting that Rev binding to Sam68 and to CRM1 may occur independently or simultaneously. Subsequent interaction of CRM1 with several nucleoporins, including CAN/Nup214 and Nup98, has been shown to recruit the complex containing Rev, RanGTP, and RRE-containing RNAs from the nucleus to the nuclear pore (17, 43, 79). Nevertheless, we currently have no information concerning the molecular events of Sam68 involvement with the complex of CRM1, Rev, RanGTP, and RRE-containing mRNAs. In particular, we do not know how Sam68 dissociates itself from the complex prior to translocation of the complex through the nuclear pore. Thus, further studies to understand the precise roles of Sam68 in the CRM1-mediated Rev nuclear export pathway are warranted.

In summary, our results obtained from the present study,

i.e., the inhibition of HIV-1 gene expression and viral replication, Rev-dependent gene expression, and CRM1-mediated Rev nuclear export by Sam68 down-modulation, along with our previous findings that Sam68 expression is able to alleviate a Rev function block in astrocytes through direct interaction and enhanced Rev nuclear export (35), support a model (Fig. 7) in which Sam68 binds to the complex of Rev, RRE-containing RNAs, CRM1, and RanGTP, and facilitates transport of the complex to the NPC, within close proximity to the cytoplasmic side of the nuclear membrane, where the complex is docked by direct binding of CRM1 to nucleoporins, followed by the release of CRM1, Rev, and RRE-containing RNAs into the cytoplasm.

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