Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 1607–1612, February 1998
Cell Biology

Interaction of the HIV-1 Rev cofactor eukaryotic initiation factor 5A with ribosomal protein L5

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Edited by Peter K. Vogt, The Scripps Research Institute, La Jolla, CA, and approved December 12, 1997 (received for review June 27, 1997)

ABSTRACT **It has previously been shown that interaction of eukaryotic initiation factor 5A (eIF-5A) with the Rev trans-activator protein of HIV-1 mediates the transport of unspliced or incompletely spliced viral mRNAs across the nuclear envelope. Consequently, mutants of eIF-5A block Rev function and thereby replication of HIV-1 in trans, indicating that eIF-5A is a crucial protein that connects the viral Rev regulator with cellular RNA transport systems. Here we show that the ribosomal protein L5, which is the central protein component of the 5S rRNA export system, is a cellular interaction partner of eIF-5A. Functional studies demonstrate that overexpression of L5 protein significantly enhances Rev activity. Furthermore, Rev nuclear export activity is inhibited in human somatic cells by antibodies that recognize eIF-5A or L5. Our data suggest that the Rev export pathway shares components of a cellular transport system involved in the intracellular trafficking of polymerase III (5S rRNA) transcripts.**

The transport of proteins and ribonucleoprotein (RNP) particles into and out of the cell nucleus is mediated by nuclear pore complexes, which are an integral part of the nuclear envelope. Over the past few years, significant advances in the understanding of receptor-mediated nuclear import of proteins have been made (for reviews, see refs. 1–3). In contrast, the mechanisms mediating the transport of RNA, although expected to exploit similar processes, are still poorly understood. Nevertheless, several candidate proteins conceivably involved in RNP-mediated export of RNA have been identified (4). Among them are nucleocytoplasmic shuttle proteins such as the heterogeneous nuclear RNP A1 protein that is presumably involved in the export of mRNA, glyceraldehyde-3-phosphate dehydrogenase that is possibly involved in the transport of tRNA, and also proteins mediating the export of 5S rRNA, such as the transcription factor IIIA (TFIIIA) or ribosomal protein L5. Furthermore, certain viral proteins have been described as affecting the intracellular distribution of viral mRNA. In particular, the regulatory protein Rev of HIV-1 appears to be a specific RNA export factor (5).

The activity of Rev is essential for HIV-1 replication. In the absence of Rev, only fully spliced HIV-1 mRNAs, encoding viral regulatory proteins, accumulate in the cytoplasm. In the presence of Rev, incompletely spliced and unspliced viral transcripts, encoding the viral structural proteins or serving as progeny virus genomes, are transported across the nuclear envelope (6–9). In accordance with its activity in RNA export, Rev has been shown to be a nucleolar protein that shuttles constantly between the nucleus and cytoplasm (10–12). A series of studies demonstrated that Rev binds in a sequence-specific manner to the Rev response element (RRE) (13–16), a cis-acting target sequence within the *env* gene (17, 18), resulting in the transport of viral mRNA independent of pre-mRNA splicing (19).

Functional analyses of the Rev protein revealed a leucine-rich carboxyl-terminal activation domain that is required for interaction with cellular cofactors (20–23) and acts as a nuclear export signal (NES; for reviews, see refs. 24 and 25). In fact, various proteins that are able to bind to this region have been described, including eukaryotic initiation factor 5A (eIF-5A) (26), the nucleoporin-like protein human Rev interacting protein (hRIP)/Rab (27, 28) and, more recently, the nuclear pore-associated factor CRM1 (29) that is critically involved in the translocation of NES-containing proteins through the nuclear pore complex (30–33). In particular, eIF-5A has been recently shown to directly influence the nuclear export of Rev and thereby virus replication (34, 35). These data suggested that eIF-5A is part of, or provides access to, a cellular nucleocytoplasmic RNA transport system.

The purpose of this study was to identify nuclear eIF-5A binding partners to further elucidate the transport pathway that is exploited by the HIV-1 Rev protein for viral RNA export.

MATERIALS AND METHODS

Yeast Two-Hybrid Assay. Maintenance and transformation of yeast cells and screening of a HeLaS3 cell-derived cDNA library using the Matchmaker two-hybrid system (CLONTECH) was performed by following the manufacturer's protocol. The plasmid encoding a GAL4 binding domain–eIF-5A fusion protein (pGB5A) was constructed by ligating a 489-bp *Eco*RI–*Bam*HI fragment obtained from peIF-5A (26) by PCR technology (36) between the *Eco*RI and *Bam*HI sites of the yeast expression vector pGBT9 (CLONTECH). The coding region of the resulting plasmid was confirmed by DNA sequencing using a Applied Biosystems model 373A automatic sequencer with the Prism Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

Plasmids and DNA Transfection. The pL5 expression plasmid was generated by cloning the human L5 coding region as a 980-bp fragment between the *Hin*dIII and *Bam*HI sites (blunt ends) of the pBC12/CMV vector (37). Approximately 2.5 ×

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Abbreviations: RNP, ribonucleoprotein; RRE, Rev response element; eIF-5A, eukaryotic initiation factor 5A; GST, glutathione *S*-transferase; NES, nuclear export signal; hRIP, human Rev interacting protein.

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10^5 COS cells were transfected with 500 ng of the expression plasmids peIF-5A (26) or pL5 by using DEAE-dextran and chloroquine as described (38). Vectors for *in vitro* transcription with T7 polymerase encoding L5 (p3L5) or the L5 antisense strand (p3L5as) were constructed by cloning the respective sequences between the *Bam*HI and *Eco*RI sites of the vector pcDNA3 (Invitrogen). The plasmids pGEX-Rev, pGEX-RevM32, pGEX-eIF-5A, pGEX-eIF-5AM13, and pGEX-eIF-5AM14 are bacterial expression plasmids that express Rev or eIF-5A fused to the carboxyl terminus of glutathione *S*-transferase (GST) (34). Plasmids expressing either GST–L5 or His-tagged L5 fusion proteins were constructed by standard methods using synthetic double-strand oligonucleotides with optimal *Escherichia coli* codon usage and the bacterial expression vectors pGEX-3X (Pharmacia) and pTrcHisC (Invitrogen), respectively. For provirus rescue assays, 2.5×10^5 COS cells were cotransfected as described above with a mixture containing 100 ng of HIV-1Δ*rev* proviral DNA (39), 50 ng of pcREV (40), 1 μg of pL5, and 100 ng pBC12/RSV/SEAP expression plasmid (internal transfection efficiency control, ref. 41). In transfections without pcREV or pL5, total input DNA was kept constant by inclusion of the parental expression vector pBC12/CMV as a negative control.

Antibodies. The anti-L5 human autoimmune serum (α-L5) has been described (42). eIF-5A-specific rabbit IgG (26) was affinity-purified on recombinant eIF-5A coupled to Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Carboxyl-terminal peptides of L5 (NH_2-CAQKKASFL-RAQERAAES-COOH) (43) and hRIP/Rab (NH_2-CTGQF-PTGSSSTNPFL-COOH) (28) were conjugated to BSA and used to immunize rabbits as described (26). Affinity purification was carried out as described above. Both antibodies specifically immunoprecipitated the respective antigen from protein extracts of transfected COS cell cultures and reticulocyte extracts. Indirect immunofluorescence studies using the anti-peptide L5 antibody demonstrated the typical nuclear/nucleolar and faint cytoplasmic staining for L5 protein (44) in HeLa cells (data not shown).

Coupled *in Vitro* Transcription/Translation and Coprecipitation Studies. Reactions were carried out using the TNT system (Promega) with [^{35}S]cysteine (Amersham). Twenty microliters of the reaction product was incubated on a rotator with affinity-purified anti-eIF-5A antibody (1:100 dilution) or human anti-L5 antiserum (42) (1:100 dilution) in 200 μl of 10 mM Tris·HCl, pH 7.5/150 mM NaCl/1% sodium deoxycholate ≈/0.1% SDS/0.5% Triton X-100 at 4°C for ~15 h. Subsequently, 10 μl of protein A-agarose was added, and the resulting mixture was incubated for another hour. The immune complexes were washed twice with 500 μl of the same buffer, and the precipitated proteins were resolved on SDS/12% polyacrylamide gels and visualized by autoradiography.

For coimmunoprecipitation studies using total cell lysates, 5×10^6 HeLa cells were collected with a cell scraper and washed twice with ice-cold PBS. The cells were resuspended in 500 μl of ice-cold Hepes buffer [10 mM Hepes, pH 7.3/5 mM KCl/1.5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride/1 μM pepstatin/chymostatin (5 μg/ml)/leupeptin (10 μg/ml)], homogenized on ice with 30 strokes of a Wheaton Dounce homogenizer (1 ml, tight pestel), and then centrifuged for 10 min at 10,000 × *g* at 4°C to remove cell debris. The supernatant (400 μl) was diluted with Hepes buffer to a total volume of 600 μl. Equal volumes (200 μl) were then distributed into three sample tubes. The lysates were precleared at 4°C with 20 μl of protein A-Sepharose CL-4B (Pharmacia) equilibrated with Hepes buffer at 4°C. Samples were then incubated at 4°C for 1 h with 5 μg of rabbit preimmune IgG, 5 μg of rabbit polyclonal anti-L5 IgG (α-pL5), or 5 μg of rabbit polyclonal anti-eIF-5A IgG (α-eIF-5A). After subsequent addition of 20 μl of protein A-Sepharose CL-4B, the samples were incubated for another hour. Precipitated immune complexes were finally washed three times with 500 μl of Hepes buffer and the proteins were resolved on SDS/10% polyacrylamide gels for subsequent L5 protein-specific Western blot analysis. Proteins were detected with a specific rabbit anti-L5 primary antibody (α-pL5) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) and the ECL-Western blotting detection kit (Amersham) according to the manufacturer's instructions.

Expression of Recombinant Fusion Proteins. GST fusion proteins were expressed in *E. coli* BL21. The fusion proteins were purified from crude lysates by affinity chromatography on glutathione-Sepharose 4B according to the manufacturer's protocol (Pharmacia). The integrities of the eluted GST fusion proteins were confirmed by Western blot analysis and the final protein preparations were concentrated by ultrafiltration with a PM10 filter device (Amicon) and stored at −70°C.

One-liter cultures of *E. coli* (BL21) cells expressing His-tagged fusion proteins were lysed by a combination of lysozyme treatment (Boehringer Mannheim) and sonification. After lysis, the bacterial extracts were centrifuged to remove cell debris and ribosomes for 30 min at 100,000 × *g* at 4°C resulting in S100 supernatants.

***In Vitro* Binding of Recombinant Fusion Proteins.** Two hundred microliters of bacterial lysates (S100 supernatants) containing either His-tagged human ribosomal protein L5 or His-tagged prolactin were incubated for 30 min at room temperature with 5 μg of the GST–eIF-5A or 5 μg of control fusion proteins, respectively. The binding reactions were then mixed with 20 μl of Talon metal affinity resin (CLONTECH) and incubated for another 30 min. Subsequently, the complexes were washed four times with 500 μl of phosphate buffer [50 mM sodium phosphate, pH 8.0/300 mM NaCl/1 mM phenylmethylsulfonyl fluoride/1 μM pepstatin/chymostatin (5 μg/ml)/leupeptin (10 μg/ml)]. The bound complexes were then either eluted with phosphate buffer containing 500 mM imidazole or boiled directly in SDS gel loading buffer. The eluted proteins were resolved on SDS/12.5% polyacrylamide gels and subjected to GST-specific Western blot analysis as described above.

Microinjection Studies. HeLa cells (HeLaneoRRE) constitutively expressing the HIV-1 RRE sequence (45) were microinjected into the nucleus, and indirect immunofluorescence was performed essentially as described (34) with mouse monoclonal antibodies to Rev or GST. GST–Rev (0.8 mg/ml) or GST–RevM32 (0.8 mg/ml) and rabbit IgG (0.5 mg/ml) were injected in combination with rabbit polyclonal anti-eIF-5A (α-eIF-5A; 1.0 mg/ml), rabbit polyclonal anti-L5 (α-pL5; 3.0 mg/ml), rabbit polyclonal anti-hRIP/Rab (α-phRIP/Rab; 5.0 mg/ml), or 10% anti-L5 human autoimmune serum (α-L5) (42). For antigen competition, the L5-derived peptide was included in the microinjection at a concentration of 0.3 mg/ml.

RESULTS

Interaction of eIF-5A with Ribosomal Protein L5. As an initial approach to identifying putative binding partners of eIF-5A, we used the yeast two-hybrid system (46) to screen a HeLaS3 cDNA library (CLONTECH). This assay makes use of the fact that the separate DNA binding and activation domains of the yeast GAL4 trans-activator are not independently functional. However, the activation potential of the GAL4 trans-activator may be restored when its two domains are brought into close proximity to each other by means of heterologous protein sequences that are fused to the respective GAL4 domains. With this assay, the initial screening of 3×10^6 independent clones of the cDNA library derived from HeLaS3 cells linked to the GAL4 activation domain resulted in 314 primary (HIS^+) transformants, encoding putative proteins that are able to interact with a GAL4 binding domain–eIF-5A fusion protein. These transformants were subsequently tested

for β-galactosidase activity by using a filter (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) and a liquid (2-nitrophenyl β-D-galactopyranoside) assay. However, only weakly elevated levels of β-galactosidase activities were detectable in these assays, presumably due to competition of the respective fusion proteins with endogenous binding partners. Therefore, 50 independent library plasmids were randomly isolated from the yeast transformants and directly sequenced. However, most of these sequences did not represent true coding regions (e.g., being untranslated sequences), were homologous to antisense transcripts, or were not in the correct reading frame with respect to the GAL4 activation domain. In fact, only one of the isolated sequences fulfilled all criteria to be a true positive, indicated by an uninterrupted coding region fused in-frame to the GAL4 activation domain and a requirement for the presence of the GAL4 binding domain–eIF-5A fusion protein for GAL4-specific transcriptional trans-activation in yeast (Fig. 1*A*). The entire sequence of this single candidate cDNA was subsequently determined and was found to contain a full-length copy of the gene encoding human ribosomal protein L5. The amino acid sequence deduced (Fig. 1*B*) is identical with the recently published human sequence and is closely related to the rat homologue found in the GenBank database (43, 47).

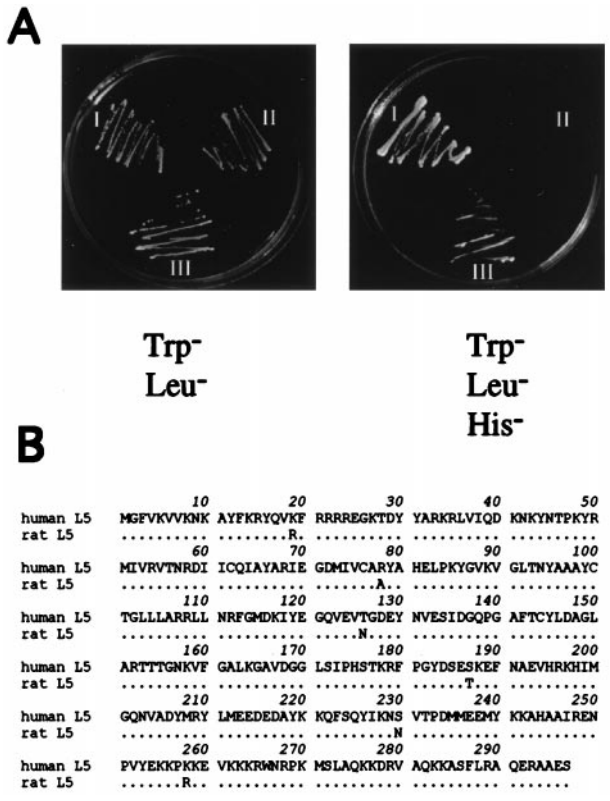

FIG. 1. Isolation of a ribosomal protein L5 cDNA by yeast two-hybrid assay. (*A*) A cDNA library derived from HeLaS3 cells was used to identify proteins that bind to human eIF-5A. Cell growth on His^- medium was observed by the interaction of the GAL4 binding domain–eIF-5A and the L5–GAL4 activation domain fusion proteins. Yeast cells were transformed with the following constructs: Regions: I (positive control), pVA3 (p53/GAL4 binding domain hybrid) + pTD1 (simian virus 40 large tumor antigen–GAL4 activation domain hybrid); II (negative control), pGB5A (eIF-5A–GAL4 binding domain hybrid) + pGAD424 (GAL4 activation domain); III, pGB5A + pGADL5 (L5–GAL4 activation domain hybrid). (*B*) Amino acid sequence of ribosomal protein L5. The isolated cDNA encodes a protein that perfectly matches the recently published sequence and displays strong homology to the L5 protein from rat (43, 47) (indicated on the lower line).

To demonstrate the interaction of eIF-5A with the L5 protein in an independent assay, *in vitro* translated L5 protein was subjected to immunoprecipitation analysis with two specific antibodies to detect eIF-5A or L5. Neither an affinity-purified polyclonal rabbit anti-eIF-5A antibody nor the L5-specific human autoimmune antiserum (42) cross-reacted with either eIF-5A or L5 protein, as shown by Western blot analysis in Fig. 2*A*, lanes 1 and 3. Even when eIF-5A or L5 sequences were overexpressed in COS cells, we failed to detect any cross-reactivity in the respective protein extracts (Fig. 2*A*, lanes 2 and 4). Note that the anti-L5 serum recognizes L5 signals of ~36 kDa and, less intensely, of ~33 kDa (Fig. 2*A*, lanes 3 and 4). The latter band may originate from the use of an alternative internal translational start site in the L5 mRNA (43).

We next prepared radiolabeled protein extracts by coupled *in vitro* transcription/translation. Without addition of DNA, both the eIF-5A-specific and the L5-specific antibodies failed to precipitate a distinct radiolabeled protein in control experiments (Fig. 2*B*, lanes 2 and 5). The same result was obtained by expressing full-length L5 antisense sequences in this system (Fig. 2*B*, lanes 1 and 4). However, when L5 protein was synthesized, the eIF-5A-specific antibodies precipitated two radiolabeled proteins of ≈35 kDa and ≈33 kDa (Fig. 2*B*, lane 3), resembling the signals detected in cellular protein extracts with the anti-L5 serum (Fig. 2*A*, lanes 3 and 4). Obviously, this data suggested that anti-eIF-5A antibodies are able to coprecipitate L5 protein due to eIF-5A–L5 interaction. This is possibly due to the fact that the reticulocyte extracts used are a rich source of the highly conserved eIF-5A protein, as demonstrated by Western blot analysis (data not shown). As expected, the anti-L5 serum precipitated the L5 protein (Fig. 2*B*, lane 6). Importantly, the anti-eIF-5A antibodies failed to precipitate L5 protein when precleared extracts (depleted of L5 protein by using the anti-L5 serum) were used (Fig. 2*B*, lane 7).

Next we were interested in whether anti-eIF-5A antibodies are also able to coprecipitate L5 from a total cell lysate. For this, equal amounts of HeLa cell lysates were immunoprecipitated with preimmune IgG (negative control), anti-eIF-5A (α-eIF-5A), or anti-L5 (α-pL5; positive control) antibody. The precipitated proteins were subsequently resolved on SDS/polyacrylamide gels, blotted, and subjected to L5-specific Western blot analysis. As shown in Fig. 3*A*, lanes 2 and 3, anti-eIF-5A antibodies clearly coprecipitated L5 protein from these cellular extracts.

Finally, we tested the binding of L5 to wild-type and eIF-5A mutant proteins *in vitro.* Bacterial extracts (S100 supernatants) containing either recombinant His-tagged L5 or His-tagged prolactin (48) (negative control) were incubated together with various GST fusion proteins. The binding reactions were then mixed with metal affinity resin and washed, and the bound complexes, containing the His-tagged proteins, were eluted and subjected to Western blot analysis with anti-GST antibody. The data obtained clearly demonstrated that wild-type eIF-5A, as well as the mutant proteins eIF-5A-M13 and eIF-5A-M14, interacted with L5 protein in this assay (Fig. 3*B*, lanes 3–5). Note the decreased mobility of eIF-5A-M14 (Fig. 3*B*, lanes 5 vs. 3), which is a typical feature of this mutant protein (O.R., unpublished observation). As expected, control experiments using either the unrelated GST–M9 protein, which contains the nuclear import and export signals of the heterogeneous nuclear RNP A1 protein (49, 50) (Fig. 3*B*, lane 2), or His-tagged prolactin (Fig. 3*B*, lanes 6–9) failed to produce an eIF-5A-specific signal. Furthermore, the presence of equal amounts of L5 protein in the various binding reactions have been confirmed by Western blot analysis with anti-L5 antibody (data not shown).

Thus, these analyses confirmed the eIF-5A–L5 interaction, initially identified by the yeast two-hybrid system.

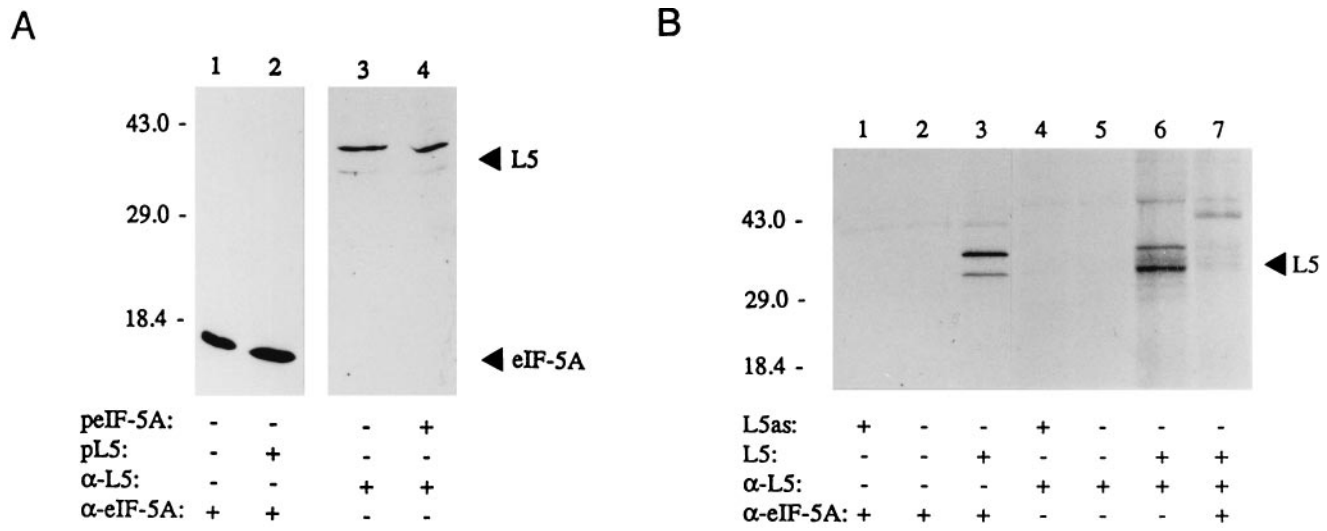

FIG. 2. Interaction of ribosomal protein L5 with eIF-5A in reticulocyte extracts. (*A*) Binding specificity of affinity-purified rabbit polyclonal anti-eIF-5A antibodies (α-eIF-5A; lanes 1 and 2) and human anti-L5 protein antiserum (α-L5; lanes 3 and 4) (42). Protein extracts of untransfected COS cells and cultures transiently transfected with peIF-5A (26) or pL5 were subjected to Western blot analysis. Both the anti-eIF-5A and anti-L5 antibodies show high specificity for their homologous antigen. Molecular mass standards (in kilodaltons) are indicated on the left. The location of L5 and eIF-5A protein are indicated on the right. (*B*) Coprecipitation of L5 protein with anti-eIF-5A antibody. Sequences encoding either the human L5 gene (L5) or the corresponding antisense sequence (L5as) were expressed in an *in vitro* transcription/translation system, followed by immunoprecipitation with anti-eIF-5A or anti-L5 antibodies shown in *A*. The reticulocyte extract used in lane 7 was first depleted of L5 protein using the anti-L5 serum and then subjected to eIF-5A-specific immunoprecipitation.

Effect of L5 Protein on HIV-1 Rev Activity. We next wanted to investigate whether L5 expression does indeed affect HIV-1 Rev function. To test this, we transfected COS cells as described (28, 38) with a mixture of HXB-2-derived Rev-deficient proviral DNA (HIV-1Δ*rev*) (39) and expression plasmids for Rev and L5 protein. Rev-mediated HIV structural gene expression was assayed ≈60 h after transfection by determining p24 Gag protein levels in the cell supernatants. Expression of secreted alkaline phosphatase (41) served as an internal transfection control to normalize the p24 assays. As expected and shown in Table 1, Rev trans-activation rescued p24 expression of the Rev-deficient provirus whereas overexpression of the human L5 gene by itself had no such effect. In contrast, coexpression of Rev and the ribosomal protein L5 did result in a significant enhancement of Rev activity by ≈130%.

The rapid nuclear export of Rev was recently shown to be mediated by the Rev activation domain (24). Therefore, we also investigated the effect of various antibodies on the export of GST–Rev protein after microinjection into HeLa cell nuclei. For this we generated a set of affinity-purified anti-peptide antibodies that specifically recognize the L5 protein (α-pL5) or the proposed Rev activation domain binding factor hRIP/Rab (α-phRIP/Rab). Furthermore, the site of injection into the HeLa cells was internally controlled by detection of the coinjected immunoglobulins, and nuclei were visualized by DNA staining (Fig. 4). Nuclear injection of GST–Rev wild-type protein resulted in transport of the protein to the cytoplasm (Fig. 4*A*), indicating that nuclear export of Rev is more efficient than its nuclear localization signal-mediated reimport, a finding that was also demonstrated in *Xenopus* oocytes (51). Control experiments confirmed that the activation domain mutant GST–RevM32 remained in the nucleus (Fig. 4*B*). However, coinjection of GST–Rev and the affinity-purified anti-L5 or anti-eIF-5A antibody (Fig. 4 *C* and *F*, respectively) or the anti-L5 serum (Fig. 4*E*) resulted in inhibition of Rev export. In contrast, the affinity-purified anti-hRIP/Rab antibody had no effect on Rev translocation (Fig. 4*G*) in these experiments. These data are not surprising in the light of the findings that deletion of the gene encoding the hRIP/Rab homologue in yeast (*RIP1*) has only a marginal effect on Rev trans-activation and does not affect the nuclear export of NES-containing export substrates (30, 52). Importantly, the blocking effect of the α-pL5 antibody on Rev export could be released when the respective antigen was coinjected (Fig. 4*D*). Finally, the injection of GST–L5 protein into the cell nucleus resulted, as expected, in its nuclear export (Fig. 4*H*).

DISCUSSION

In this study we describe experiments that identify ribosomal protein L5 as a cellular interaction partner of eIF-5A. The L5 protein was originally described as an ~35-kDa protein that participates in the assembly of 5S rRNA into ribosomes and appears to concentrate in the nucleolus (44). Moreover, the L5 protein is the single protein moiety of the 7S RNP that contains the vast majority of all cellular nonribosomal-associated 5S rRNA. Importantly, it was demonstrated in *Xenopus* oocytes that, the 7S RNPs containing either L5 or the 5S rRNA-specific transcription factor TFIIIA as protein moiety migrate out of the nucleus and accumulate in the cytoplasm (53). Mutant 5S rRNAs that were impaired in their ability to bind either L5 or TFIIIA were retained in the cell nucleus. Thus, L5 protein appears to be a central part of a fundamental cellular RNA export pathway that is involved in the translocation of polymerase III transcripts (5S rRNA) across the nuclear envelope (4). In agreement with this, L5 appears to contain distinct nuclear import (54) and presumably export signals.

Our findings suggest that HIV-1 Rev exploits components of the cellular 5S rRNA export pathway for nucleocytoplasmic trafficking of incompletely spliced viral mRNAs. Indeed, previous microinjection studies revealed that saturation of the Rev export pathway also resulted in an almost complete block in the export of 5S rRNA in oocytes (55). Clearly, our data are able to provide a straightforward explanation for these findings, suggesting that Rev is exported via eIF-5A–L5 interaction. In this model, the pool of unspliced or singly spliced RRE-containing viral mRNAs, which are poorly spliced due to inefficient HIV-1 splice sites (56), is retained in the nucleus until these RNAs are eventually degraded (6) or spliced and exported via a general mRNA export pathway. However, when Rev is present, it binds to (13–16) and multimerizes (57, 58) on the viral RRE RNA. Subsequently, this complex interacts with

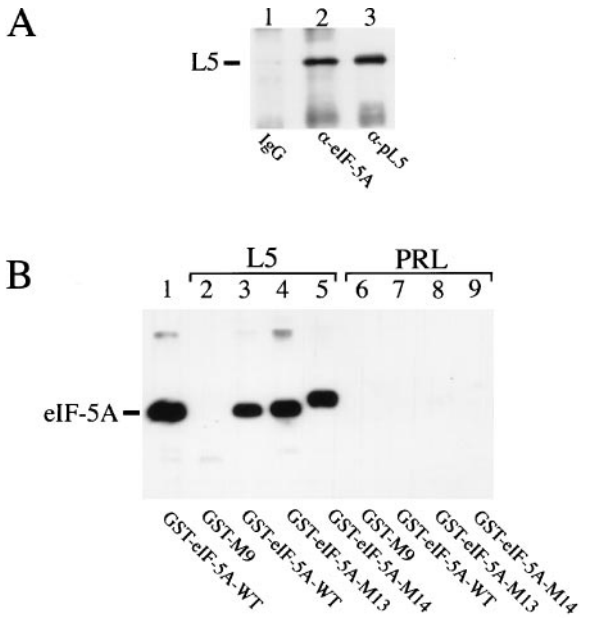

FIG. 3. Binding of L5 to eIF-5A *in vivo* and *in vitro*. (*A*) Coprecipitation of eIF-5A and ribosomal protein L5 using total cell lysate. Equal amounts of HeLa cell extracts were subjected to immunoprecipitation analyses with preimmune IgG (lane 1, negative control), anti-eIF-5A antibodies (lane 2, α-eIF–5A), or anti-L5 antibodies (α-pL5, lane 3, positive control). The precipitated complexes were resolved on SDS/polyacrylamide gels, immobilized on membranes, and subjected to Western blot analysis with anti-L5 antibodies (α-pL5). The location of L5 protein is indicated on the left. (*B*) Specific interaction of recombinant His-tagged L5 with GST–eIF-5A fusion proteins. Bacterial S100 extracts containing either His-tagged L5 (lanes 2–5) or His-tagged prolactin (PRL, lanes 6–9) protein were incubated *in vitro* with various GST fusion proteins (indicated at the bottom). The binding reactions were then immobilized with metal affinity resin and eluted with imidazole. Eluted proteins were separated on SDS/polyacrylamide gels, blotted, and subjected to Western blot analysis with anti-GST antibodies. For comparison, recombinant GST–eIF-5A wild-type protein (WT) was loaded directly in lane 1 of the SDS/polyacrylamide gel (indicated at the left). The GST–M9 (49, 50) protein served as negative control (lanes 2 and 6).

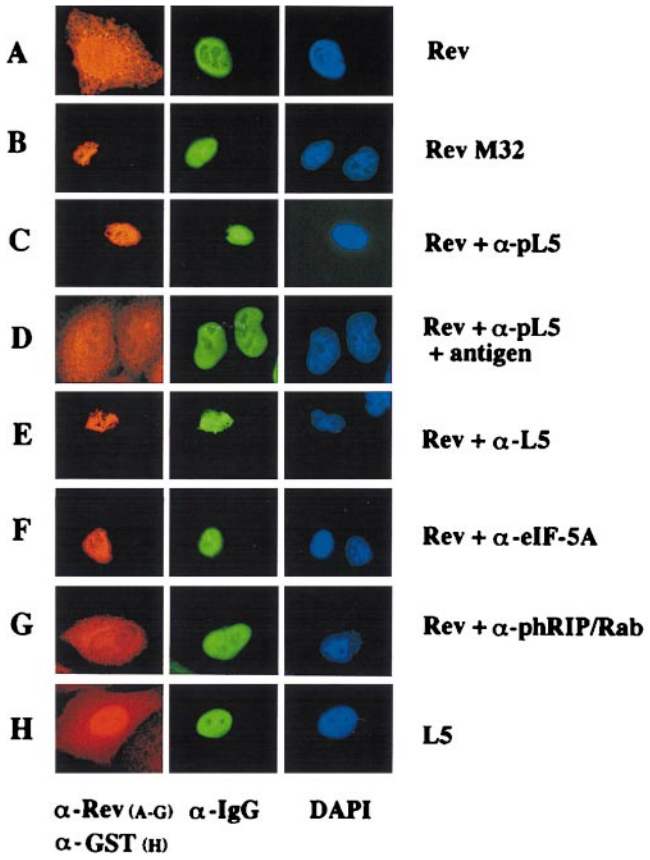

FIG. 4. Rev export in human somatic cells. Nuclei of HeLa cells were microinjected with the indicated GST Rev (*A–G*) or GST L5 (*H*) fusion proteins, antibodies, and rabbit IgG. About 20 min after microinjection, the cells were fixed and subjected to double-label indirect immunofluorescence analysis [Texas Red, Rev or L5; fluorescein isothiocyanate, IgG (internal injection control)], and nuclei were visualized by DNA staining (4′,6-diamidino-2-phenylindole). Control experiments using Rev or RevM32 (Rev activation domain mutant) confirmed the requirement of the activation domain for Rev nuclear export (*A* and *B*).

eIF-5A, thereby accessing the 5S rRNA export pathway via L5 interaction. In this model, eIF-5A would act as an adaptor that allows viral mRNAs to access the specific 5S rRNA nuclear export system. However, our findings that overexpression of L5 enhances Rev activity (Table 1) and that eIF-5AM13 and eIF-5AM14, both mutant proteins that have previously been shown to inhibit Rev-mediated nuclear export (34), still bind to L5 (Fig. 3*B*), also allows an alternative functional interpretation of the eIF-5A–L5 pathway. In this model, L5 is conceived to facilitate efficient nuclear accumulation of eIF-5A, either by enhancing eIF-5A import or by mediating its nuclear retention. In the nucleus, eIF-5A might then be involved not only in the export of HIV mRNAs but also perhaps in the TFIIIA-mediated translocation of 5S rRNA. Because TFIIIA contains a region that may be functionally equivalent to the Rev activation domain (55, 59), saturation of the Rev pathway would then indeed also explain the previously reported block in 5S rRNA export (55). Moreover, in this model, overexpression of L5 protein would be expected to increase the nuclear pool of eIF-5A, thereby providing higher nuclear levels of Rev cofactor that, in turn, would result in enhanced Rev activity; this was indeed observed in this study (Table 1). Finally, the inhibitory effect of anti-L5 antibodies on Rev nuclear export (Fig. 4 *C–E*) could be explained by inactivation of L5 protein, which might then lead to subcritical levels of nuclear eIF-5A, resulting in the export-negative Rev phenotype observed.

Table 1. Effect of L5 expression on HIV-1 structural gene expression

Vectors transfected	HIV-1 p24, pg/ml				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
HIV-1Δ*rev* + pBC12/CMV	<10	<10	<10	<10	<10
+ pcREV	39.9	41.4	40.9	54.3	40.7
+ pL5	<10	<10	<10	<10	<10
+ pcREV + pL5	96.1	105	92.9	94.2	122

COS cells (2.5×10^5 cells) were cotransfected by using DEAE-dextran and chloroquine with the indicated expression plasmids. In addition, a vector expressing constitutively secreted alkaline phosphatase was included in all transfections to serve as internal transfection efficiency control. p24 Gag and secreted alkaline phosphatase assays were performed at ≈60 h after transfection by using an ELISA or a colorimetric assay (41), respectively. Data from five experiments are shown.

In both models however, the transport of the export substrate through the nuclear pore complex is conceived to be mediated by CRM1, which appears to be a general export receptor for leucine-rich NESs (30–33). However, many cellular and viral proteins of entirely different functions have so far been identified, all of which contain this type of NES. For example, this includes IκB (60), which is an inhibitor of the transcription factor NF-κB, cAMP-dependent protein kinase inhibitor (61, 62) or mitogen-activated protein kinase kinase (63), and proteins with obvious activities in RNA transport

such as HIV-1 Rev (19), adenovirus E4 (64), or the 5S rRNA transcription factor TFIIIA (59). Thus, it seems rather unlikely that the nuclear export of all of these different factors is regulated identically without causing deleterious effects to cell metabolism. Therefore, it is likely that adaptor molecules are required to provide specificity by accessing different nuclear export pathways, ultimately leading to the successful translocation of the export cargo across the nuclear envelope. With respect to HIV-1 Rev export, L5 and eIF-5A are obvious candidates for such small adaptor molecules and it will be of interest to see how they interact with the general nuclear pore-associated import and export machinery. In more general terms, it is expected that future research will result in the identification of even more components of specific cellular nucleo-cytoplasmic transport pathways. In particular, it is conceived that regulation of nuclear export at the molecular level will soon become a research area as complex as the ones seen in the field of transcriptional regulation.

We thank Dr. Apostolia Guialis (The National Hellenic Research Foundation, Athens, Greece) for the generous gift of human anti-L5 antiserum, Dr. Gideon Dreyfuss (Howard Hughes Medical Institute, University of Pennsylvania) for the GST–M9 construct, and Dr. Sarah L. Thomas for critical reading of the manuscript. This work was supported in part (M.O. and O.R.) by grant of the Deutsche Forschungsgemeinschaft (SFB466).

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