Eukaryotic Initiation Factor 5A Is a Cellular Target of the Human Immunodeficiency Virus Type 1 Rev Activation Domain Mediating Trans-Activation

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Abstract. Expression of human immunodeficiency virus type 1 (HIV-1) structural proteins requires the presence of the viral trans-activator protein Rev. Rev is localized in the nucleus and binds specifically to the Rev response element (RRE) sequence in viral RNA. Furthermore, the interaction of the Rev activation domain with a cellular cofactor is essential for Rev function in vivo. Using cross-linking experiments and Bio-specific Interaction Analysis (BIA) we identify eukaryotic initiation factor 5A (eIF-5A) as a cellular factor binding specifically to the HIV-1 Rev activation domain. Indirect immunofluorescence studies demonstrate that a significant fraction of eIF-5A localizes to the nucleus. We also provide evidence that Rev trans-activation is functionally mediated by eIF-5A in Xenopus oocytes. Furthermore, we are able to block Rev function in mammalian cells by antisense inhibition of eIF-5A gene expression. Thus, regulation of HIV-1 gene expression by Rev involves the targeting of RRE-containing RNA to components of the cellular translation initiation complex.

Human immunodeficiency virus type 1 (HIV-1) encodes a trans-acting regulator of viral gene expression, called Rev, which is essential for virus replication (Feinberg et al., 1986; Sodroski et al., 1986; Sadaie et al., 1988; Terwilliger et al., 1988). It was shown that Rev function is required for the cytoplasmic expression of unspliced and singly spliced viral mRNAs encoding the viral structural proteins (Emerman et al., 1989; Hammarstjöld et al., 1989). Various modes of Rev action have been suggested including effects on the transport, stability, splicing, and translation of viral mRNA species (Chang and Sharp, 1989; Felber et al., 1989; Malim et al., 1989a; Arrigo and Chen, 1991; Kjems et al., 1991b; Lawrence et al., 1991; D'Agostino et al., 1992).

Previous studies demonstrated that Rev is a nuclear phosphoprotein which appears to accumulate in the nucleoli of expressing cells (Cullen et al., 1988; Hauber et al., 1988; Cochrane et al., 1989; Malim et al., 1989a; Cochrane et al., 1990b). Rev trans-activates through a highly structured cis-acting RNA sequence, the Rev response element (RRE), which is encoded by sequences residing in the env gene (Rosen et al., 1988; Hadzopoulou-Cladaras et al., 1989; Malim et al., 1989b). In vitro studies demonstrated that the rev gene product binds directly and specifically to its RRE RNA target sequence (Daly et al., 1989; Zapp and Green, 1989; Cochrane et al., 1990a; Daefler et al., 1990; Heaphy et al., 1990; Malim et al., 1990a; Olsen et al., 1990b). Multiple Rev interaction sites within the RRE have been reported (Kjems et al., 1991a). However, a substructure of the RRE, termed Stem–Loop IIB, has been mapped as the high-affinity Rev binding site (Bartel et al., 1991; Cook et al., 1991; Heaphy et al., 1991; Kjems et al., 1992; Tiley et al., 1992). Recently, evidence was provided that multimerization of Rev monomers on the RRE is required for biological activity (Malim and Cullen, 1991). In addition, Rev multimerization before RRE RNA binding has also been reported (Olsen et al., 1990a; Zapp et al., 1991).

Mutational analysis of the rev gene revealed a modular structure similar to transcriptional activator proteins (Ptashne, 1988). A basic domain rich in arginine residues, which maps to amino acids (aa) 33 to 46 in the 116 aa Rev protein, serves as a nuclear localization signal and is required for the sequence specific binding of viral RRE RNA.
(Malim et al., 1989a; Hope et al., 1990; Olsen et al., 1990a; Böhmlein et al., 1991; Kjems et al., 1991b, 1992; Malim and Cullen, 1991; Zapp et al., 1991). Amino acid residues flanking this domain are reported to be involved in the oligomerization of the Rev molecule (Olsen et al., 1990a; Malim and Cullen, 1991; Zapp et al., 1991). In addition, a protein activation domain which is required to mediate Rev effector functions in vivo is located between aa 78 and aa 93 (Malim et al., 1989a, 1991; Mermer et al., 1990; Venkatesh and Chinnadurai, 1990; Hope et al., 1990; Weichselbraun et al., 1992). A peptide core motif rich in leucine residues (aa 81 to 84) has been identified as the functionally critical part of this domain (Hope et al., 1991; Malim et al., 1991). Mutations in this region in Rev mutant proteins which display a trans-dominant (dominant-negative) phenotype. These data suggest that the Rev activation domain mediates Rev function in vivo by interacting with an unknown cellular host protein (Malim et al., 1989a, 1991; Mermer et al., 1990; Venkatesh and Chinnadurai, 1990; Hope et al., 1991). The identification of this cellular cofactor required for Rev function will provide insight into the molecular mechanism of Rev-mediated trans-activation in the course of HIV-1 infection.

We used biochemical methods to detect a cellular protein which localizes to the nucleus and interacts specifically with the activation domain of the HIV-1 Rev trans-activator protein. Subsequently, this cellular factor was identified by purification and amino acid sequencing. Finally, functional data are provided which demonstrate that this specific interaction mediates Rev trans-activation.

**Materials and Methods**

**Cross-linking Experiments and Subcellular Fractionation**

Total HeLa nuclei were prepared according to standard procedures and purified by centrifugation through a 60% sucrose cushion (Yamasaki et al., 1993). Total HeLa nuclei were prepared according to standard procedures and stored at -80°C in storage buffer (10% Sucrose, 10 mM Hepes, 10 mM NaCl, 4 mM MgCl₂, pH 6.5) as described above. After thawing, 2 μg/ml Aprotinin and 20 μg/ml Leupeptin were added to the nuclei and the suspension was centrifuged at 2,800 g for 15 min at 4°C. The pellet was washed twice in 25 mM Hepes, 150 mM NaCl, 4 mM MgCl₂, 1 mM EGTA, 10% Glycerol, 0.6% N-Octylglucoside, 2 μg/ml Aprotinin, 20 μg/ml Leupeptin, pH 7.4, and resuspended in 250 ml of the same buffer. This solution was adjusted to a final concentration of 2% NP-40 and 2% N-Octylglucoside. Solubilization of the nuclei was finally achieved using 20 strokes of a motor driven Teflon homogenizer (1,200 rpm; 4°C). The obtained homogenate was cleared by centrifugation at 100,000 g for 60 min at 4°C and the supernatant subjected to ultrafiltration using an Amicon XM 300/76 device (Amicon Corp., Danvers, MA). The resulting filtrate was then concentrated using an Amicon YM 3/76 unit.

Size exclusion chromatography on Superose 75 (Pharmacia, Upssala, Sweden) was carried out using a 16 × 1,200-mm column equilibrated with chromatography buffer (25 mM HEPES, 50 mM NaCl, 4 mM MgCl₂, 1 mM EGTA, 10% Glycerol, 0.6% N-Octylglucoside, 2 μg/ml Aprotinin, 20 μg/ml Leupeptin, pH 7.4). The protein homogenate was chromatographed at 1 ml/min and fractions were collected.

Fractions containing the 19-kD protein were pooled and loaded onto a Mono Q column (16 × 100 nm; Pharmacia) for anion exchange chromatography. Proteins were eluted by applying a salt gradient ranging from 50 mM to 1 M NaCl at a flow rate of 2 ml/min fractions containing the desired protein were pooled. After this stage of the purification scheme, two alternative separation procedures were followed.

To prepare the Rev activation domain binding protein in its native state, the technique of chromatofocusing was applied. A Mono P column (high resolution [HR]; 10 × 300 mm; Pharmacia) was equilibrated with chromatography buffer and the protein sample was loaded using a flow rate of 1 ml/min. A self-forming pH gradient was created by titrating the sample containing Mono P matrix at pH 7.4 with elution buffer (Pharmacia). Buffer pH 7.4 diluted 1:20 with chromatography buffer lacking Hepes). The obtained native protein was subjected to two-dimensional (2D) gel electrophoresis for determination of purity and identity confirmation.

For sequencing, the Rev binding protein was prepared by reversed phase chromatography using a Vydac C4 column (4.6 × 150 mm). Separation was achieved by applying a gradient of water/0.1% Trifluoroacetic Acid (TFA) to acetonitrile/0.1% TFA.

**Amino Acid Analysis and Sequence Determination**

Approximately two pmol of the purified Rev binding protein were gas-phase hydrolyzed with 6 N HCl containing 1% phenol at 110°C for 20 h on a pico tag workstation (Waters Associates, Milford, MA) and subsequently dried. Amino acid derivatives were formed by automated pre-column reaction with O-phthalaldehyde and FMOC. The amino acid derivatives were analyzed by reversed phase HPLC on a model HP 1090 M liquid chromatography using a Vydac C4 column (4.6 × 150 mm). Separation was achieved by applying a gradient of water/0.1% Trifluoroacetic Acid (TFA) to acetonitrile/0.1% TFA.

**Antiseraw**

Chemically synthesized HIV-1 Rev activation domain peptide and eIF-5A protein, which was purified as described previously (Park et al., 1986), were conjugated separately to cationized BSA (SuperCarrier™; Pierce) and conjugated to O-phthalaldehyde and FMOC. These conjugates were purified by reversed phase HPLC with a model 120A PTH amino acid analyzer (Applied Biosystems, Inc., Foster City, CA) using a C18-micro-bore column (2 × 150 mm) and fluorescence detection. Elution was carried out according to the manufacturer's recommendations.

For CNBr cleavage ~50 pmol of the isolated protein were dissolved in 100 μl of 70% formic acid containing 3 mg/ml cyanogen bromide and incubated at 20°C for 16 h in the dark. Subsequently the sample was dried and 10% of the material was used to verify the cleavage reaction by SDS-PAGE. The remainder was redissolved in TFA/acetonitrile/water (1:500:500), spotted on a polybrene coated glass filter disk and subjected directly to sequence determination.

Automated amino acid sequence analysis was performed on a model 470A sequencer. Amino acid derivatives were analyzed on-line by reversed phase HPLC with a model 120A PTH amino acid analyzer (Applied Biosystems, Inc., Foster City, CA) using a C18 micro-bore column (2 × 220 mm). Instrument control, data collection, and analysis was performed using a model 900A controller (Applied Biosystems, Inc.).

**Antiseraw**

Chemically synthesized HIV-1 Rev activation domain peptide and eIF-5A protein, which was purified as described previously (Park et al., 1986), were conjugated separately to cationized BSA (SuperCarrier™; Pierce) according to the manufacturer's protocol.

Female BALB/c mice received 50 μg conjugated Rev activation domain peptide (NH₂-LLPLELRTLDCNEDCGTSG-OH) with complete adjuvant intraperitoneally (ip) and subcutaneously (sc) on day 0. The same amount was administered in Freund's incomplete adjuvant on day 21. Two animals received intraperitoneally 20 μg conjugated eIF-5A protein in saline on day 36.

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IgG and IgM antibodies were detected in the sera by an ELISA system as previously described (Bahr et al., 1985).

To raise polyclonal anti-eIF-5A antibodies, glutathione-S-transferase-eIF-5A fusion protein was prepared as previously described (Smith and Johnson, 1988) and conjugated to cationized BSA. A rabbit was immunized on day 0 with 200 μg of this material after mixing with Freund's complete adjuvant. The rabbit was boosted by additional injections on day 8, 15, and 22 and the responses rapidly demonstrated a high level of specific anti-eIF-5A antibody as measured by immunoprecipitation of eIF-5A protein.

**Biospecific Interaction Analysis (BIA)**

Biospecific Interaction Analysis (BIA) was performed on a BIAcore™ processing unit (Pharmacia) according to the manufacturer's system manual. The eIF-5A protein used in these studies was purified as described previously (Park et al., 1986). The immunoefficiency purified anti-Rev activation domain antibodies were prepared as described previously (Kristiansen, 1978) by passing 1.5 ml of high titred pooled mouse antiseraum diluted with PBS (1:3) through a Rev-Sepharose column (5 × 20 mm). Bound mouse antiserum was eluted at 0.1 M Glycine/HCl buffer and neutralized immediately after desorption. eIF-5A and the immunoefficiency purified anti-Rev antibody were immobilized on the BIAcore chip surface at a surface concentration of 15 ng/mm². Antiserum from one mouse designated P3 was diluted 1:20 with PBS and subsequently 5–20 μl were used for serum sample analysis. Preincubation experiments were carried out by exposing the mouse antiserum (P3) to 20 μg/ml of eIF-5A or BSA for 30 min at ambient temperature before BIA.

**Plasmid Constructions**

The pcRev, pBC12/CMV, pBC12/CMV/II-2, and pDM128/CMV constructs and the plasmids encoding the Rev mutants M10, M20, and M32 have been described in detail previously (Cullen, 1986; Malim et al., 1988, 1989a, 1991). The RRE-deficient plasmid pDM128/CVMVARRE was constructed by cloning the 1.7-kb XbaI–BglII fragment from the Rev-specific CAT reporter construct pDM128 (Hope et al., 1990) between the Sall and HindIII site of pcAT (Malim et al., 1988). Substitution of the II-2 specific sequences in pBC12/CMV/II-2 (Cullen, 1986a) by the 3.7-kb HindIII–BamHI fragment of pSV-β-Galactosidase (Promega, Madison, WI) resulted in the construct pBC12/CMV/βGal. The gene encoding eIF-5A (Smit-McBride et al., 1999a) was isolated from a human cDNA as a 0.5-kb HindIII–BamHI fragment by PCR technology, using the following primers to introduce terminal HindIII and BamHI sites, respectively: sense 5'-AAG-CTTGCCGAGTGGATAGCAAGCCTCTT-3'; anti-sense 5'-GGATCC-CCTGGAGCCAGTTATTTICd2C-3'. The PCR conditions included 30 cycles of denaturation at 95°C for 1 rain, primer annealing at 60°C for 2 rain, and primer extension at 73°C for 2 min. The reaction product was digested by HindIII and BamHI and subsequently inserted in the expression vector pBC12/CMV to generate pepIF-5A. Plasmid pepIF-5Asa expresses the antisense strand of the full-length human eIF-5A cDNA.

**Immunofluorescence**

Indirect immunofluorescence studies were performed as previously described (Malim et al., 1989a) to localize the eIF-5A protein within transfected and wild-type COS cells. The primary rabbit anti-eIF-5A antibody was used at 1:100 dilution. The secondary antibody, rhodamine-conjugated goat anti-rabbit IgG, was used at a 1:50 dilution.

For confocal laser scanning microscopy COS cells were fixed using 3.7% formaldehyde/2% sucrose in PBS and permeabilized with 0.5% Triton X-100 in PBS. The cells were incubated with anti-eIF-5A antiseraum at a 1:500 dilution in PBS containing 1% BSA. To label the nuclear envelope, a 1:100 dilution of mouse mAb (CHON 211) that specifically binds to components of the nuclear pore complex (Park et al., 1987) was included in the primary antibody incubations. CHON 211 ascites was used at a 1:2000 dilution. The secondary antibodies were FITC-conjugated goat anti-mouse IgM and Texas red–conjugated goat anti–rabbit IgG (diluted 1:100 in PBS/BSA). All antibody incubations were carried out for 1 h at ambient temperature.

Two color images were obtained in the dual channel mode with a BioRad MRC 600 confocal laser scanning system (Bio Rad Laboratories, Cambridge, MA) mounted on a Zeiss IM 10 inverted microscope (Carl Zeiss Inc., Thornwood, NY). Spill-over of fluorescein into the Texas red channel was corrected for by applying the available "bleed" software. The two images were merged using the "merge" command.

**Transfections**

For indirect immunofluorescence and studies using antisense constructs, COS cells were transfected using DEAE-Dextran as previously described (Cullen, 1986b). CAT enzyme levels in protein extracts of the transfected COS cells were determined as previously described (Neumann et al., 1987).

**Microinjection Experiments**

Microinjection was performed into nuclei of stage VI Xenopus oocytes (Dumont, 1972). Typically, 15 ng of pDM128/CMV, 15 ng pcRev and 7.5 ng pBC12/CMV/βGal DNA together with 0.05 ng eIF-5A protein or 15 ng pepIF-5A DNA in 80 mN NaCl, 10 mM Hepes, pH 7.6, were coinjected into oocytes using 15 oocytes per experiment. In some experiments the pDM128/CMV reporter construct was substituted by pDM128/CVMVARRE. In all of the experiments the DNA concentration was kept constant by addition of pBC12/CMV DNA.

Incubation of the injected oocytes was carried out at 21°C for 24 h in modified Barth's medium (Gurdon and Wickens, 1983). Protein extracts were prepared by disrupting the oocytes with a pipette tip in 200 μl of 250 mM Tris-HCl, pH 7.6, and cleared by centrifugation.

CAT assays were normalized to the expression of β-galactosidase activity expressed from the internal control plasmid pBC12/CMV/βGal (Gorman et al., 1982).

**RNA Isolation and SI Nuclease Protection Analysis**

Nuclear and cytoplasmic RNAs were isolated from 100 mm cell cultures (2.5 × 10⁶ COS cells) 60 h after transfection as described previously (Malim et al., 1989b).

SI nuclease protection analysis was carried out as published previously (Malim et al., 1988). The probe used was isolated as a 629-bp SalI–EcoRI fragment from pDM128/CMV (Malim et al., 1991), fused to heterologous non-HIV-1 sequences and radioactive labeled at the SalI site using Klenow enzyme. The probe spans the HIV-1 derived splice donor site (Hope et al., 1990) and allows the input probe to be distinguished from the protected fragments corresponding to unspliced and spliced pDM128/CMV-derived RNAs.

**Results**

**Detection of the Cellular Rev Activation Domain Binding Factor**

To detect an interaction between the HIV-1 Rev activation domain and host cell proteins, we used a synthetic peptide to mimic the Rev activation domain in cross-linking experiments. The radioactive labeled cross-linker APDP (N-[4-(p-azidosalicyl-amido)butyl]-3'- (2'-pyridyl-dithio)propionamide) was coupled chemically to the Rev activation domain peptide and the resulting conjugate was incubated with total cell or cellular extracts from HeLa cells (Fig. 1). Cellular proteins interacting with the conjugated Rev activation domain were identified through activation of the cross-linker by UV-light, followed by a reductive cleavage of the cross-linked proteins. Intact nuclei from HeLa cells were incubated with Rev activation domain peptide coupled to radiolabeled N-[4-(p-azidosalicyl-amido)butyl]-3'- (2'-pyridyl-dithio)propionamide (APDP; Pierce) and subsequently cross-linked. Subfractions of labeled nuclei were subjected to SDS-PAGE and the cross-linked proteins were visualized by autoradiography. Lane 1, total nuclei; lane 2, nuclear envelope; lane 3, chromatin; lane 4, nucleoplasm; lane 5, cytoplasm. Molecular mass standards (in kilodaltons) are indicated on the left.
linked peptide-protein complexes. This protocol resulted in the transfer of the radioactive label from the cross-linker molecule onto the cellular target proteins which were subsequently subjected to gel electrophoresis and visualized by autoradiography. A typical result is shown in Fig. 1. Predominant signals migrating at a relative molecular mass of 65, 41, 39, 34, 30, 19, and 15 kD were detectable when the Rev activation domain peptide was incubated with total nuclei from HeLa cells (Fig. 1, lane J). Fractionation of the nuclei resulted in an identical cross-linking pattern for the nuclear envelope fraction (Fig. 1, lane 2 versus lane J). No signals were detected in the HeLa cell chromatin, nucleoplasmic or cytoplasmic fractions (Fig. 1, lanes 3 to 5). To demonstrate binding specificity, competition experiments were performed using unlabeled peptide. A 6-10-fold molar excess of the Rev activation domain peptide completely abrogated the cross-linking to the 19-kD protein from the envelope fraction of HeLa nuclei (Fig. 2 A, lanes 4 and 5 versus lane J). Densitometric analysis of this data revealed that the 19-kD signal is reduced by maximal 82% when compared with the nonspecific 15-kD band (not shown). In contrast, the use of a nonspecific competitor peptide derived from the HIV-1 Tat carboxyterminus (Tat aa 73 to 86) did not abolish the interaction of the HIV-1 Rev activation domain peptide with the 19-kD host protein (Fig. 2 B, lanes 2 and 3 versus lanes 4 and 5). Therefore, the band of 19 kD exhibited the highest degree of specific competition and was selected for further analysis in this study.

Purification and Identification of the Rev Binding Factor

A radiotracer isolation procedure was used to isolate the 19-kD Rev binding host protein. This approach was chosen since the detected binding factor lacks Rev binding activity when solubilized from its native nuclear context (data not shown). For this the HeLa cell nuclei were cross-linked to the Rev activation domain peptide as described above and the 19-kD target protein was purified on an analytical scale using various extraction and column purification methods (not shown). In addition, the isoelectric point was determined to be pl 5.1 by 2D gel electrophoresis using the identical material. This information was subsequently used to adjust the scheme to large-scale purification (detailed in

Figure 2. Binding specificity of nuclear proteins recognizing the HIV-1 Rev activation domain. Autoradiographs of SDS-PAGE gels are shown. The molecular mass standards (in kilodaltons) are indicated on the left. An arrow indicates the position of the 19-kD nuclear protein. (A) Cross-linking experiments using an unlabeled specific competitor (Rev activation domain peptide). The molar excess of the specific competitor used was as follows: lane J, no competitor; lane 2, onefold; lane 3, threefold; lane 4, sixfold; lane 5, 10-fold; lane 6, 50-fold. (B) Comparison of specific with nonspecific competition. A Tat-derived peptide (Tat aa 73 to 86; NH2-PTSQSRGNPTGPKQ-COOH) served as nonspecific competitor. The molar excess of the competitors used were as follows: lane J, no competitor; lanes 2 and 3, 5- and 10-fold molar excess of nonspecific competitor, respectively; lanes 4 and lane 5, 5- and 10-fold molar excess of specific competitor, respectively.

Figure 3. Purification of the Rev activation domain binding factor. 10 µg of Rev activation domain binding factor were purified from 500 g of HeLa cells. Size fractions which contained the desired protein and which were collected for further purification are shown on silver stained SDS-PAGE gels. (A) Size exclusion Chromatography. Solubilized nuclear proteins were fractionated according to size on a Superose 75 (Pharmacia) gel-filtration column. SDS-PAGE analysis of the fractions collected is shown. Molecular mass standards (in kilodaltons) are indicated on the left. (B) Anion Exchange Chromatography. The charge differences at physiological pH were exploited to separate the acidic Rev activation domain binding factor from the neutral and basic nuclear protein fractions using a Mono Q resin (Pharmacia). The 19-kD Rev cofactor eluted at a salt concentration of ~400 mM NaCl which was monitored by SDS-PAGE (lane J). (C) Separation according to isoelectric properties was achieved using Chromatofocussing on a Mono P matrix (Pharmacia). This procedure ensured the recovery of the desired protein in a native state. SDS-PAGE analysis revealed the purity of the fractions collected which eluted at a pH of 5.5 (lanes 1 and 2). (D) 2D gel electrophoresis was used to demonstrate purity and identity of the protein prepared under native conditions, pl standards for isoelectric focussing (IEF) as the first dimension are indicated on the bottom. The purified Rev activation domain binding factor is indicated by a triangle. Molecular mass standards for SDS-PAGE as the second dimension are shown on the left. (E) To prepare the protein in a solvent mixture suitable for amino acid sequence determination, reversed phase chromatography on a Vydac C4 support was performed. The 19-kD protein desorbed at an acetonitril concentration of 55%. Again, the purity of the protein was confirmed by SDS-PAGE (lanes 1 and 2).
Figure 4. Amino acid sequence determination after cyanogen bromide (CNBr) cleavage of the Rev binding factor. The amino acid sequence of the 154-amino acid (aa) eIF-5A protein is shown. The sequence of the indicated CNBr-derived peptides (boxes) gave an exact match with the published human eIF-5A sequence (Smit-McBride et al., 1989a). No sequence could be determined for aa 1 to 20 due to an amino-terminal modification in eIF-5A (Wolff et al., 1992).

To raise mAbs specific for the Rev activation domain, mice were immunized with the activation domain peptide (Rev aa 75 to 93: NH2-LPPLERLTLDCNEDCGTSG-COOH) conjugated to BSA. Interestingly, serum from one mouse designated P3, taken 7 d after a boost with the peptide–BSA conjugate (bleed day 28; Table I) displayed not only IgG antibodies to the complete Rev protein but also IgM antibodies to purified eIF-5A. This IgM response to eIF-5A was induced to switch to an IgG response after injection of the mouse on day 36 with eIF-5A protein (bleed day 47; Table I). Sera from control mice immunized with the carrier alone totally lacked antibodies with specificity against either Rev or eIF-5A proteins. In addition, all preimmune sera did not react with Rev-derived peptides, Rev or eIF-5A (data not shown).

Biospecific interaction analysis was performed using a BIAcore processing unit (Pharmacia) to investigate the binding pattern of the P3 antiserum. As expected from the antibody levels detected, eIF-5A protein immobilized on the BIAcore support-chip retained effectively P3 serum compo-

Table 1. Antibody Levels of Immunized BALB/c Mice

| Animal | Peptide† | eIF-5A‡ | Bleed day | Rev IgG§ | Rev IgM¶ | eIF-5A IgG§ | eIF-5A IgM¶ |
|--------|----------|---------|-----------|----------|----------|-------------|-------------|
| P1     | +        | -       | 28        | 1.01     | 0.10     | 0.03        | 0.06        |
| P2     | +        | -       | 28        | 1.40     | 0.11     | 0.03        | 0.08        |
| P3     | +        | +       | 47        | 0.41     | 0.08     | 0.07        | 0.36        |
| P4     | +        | -       | 28        | ND       | ND       | 0.20        | 0.31        |
| P5     | +        | -       | 47        | 0.84     | 0.04     | 0.00        | 0.09        |
| P6     | +        | +       | 28        | 0.04     | 0.02     | 0.02        | 0.07        |

Animals immunized with BSA and the preimmune sera of the animals presented showed no response to the Rev-derived peptide, Rev or eIF-5A (not shown).

† Animals were immunized using a Rev-derived peptide (Rev activation domain; Rev aa 75 to 93) on day 0 and day 21 or using eIF-5A protein on day 36. Both antigens were conjugated to cationized BSA (for details see Materials and Methods).

‡ The presented absorbance values were obtained at a 1:200 or 1:50 dilution.
Figure 5. Biospecific interaction analysis (BIA). (A) Interaction of immobilized eIF-5A with the preimmune- (P3-pi; □) and the immune-serum (P3; □) from a BALB/c mouse immunized with Rev activation domain peptide (Rev aa 75 to 93). Binding values represent the relative levels and have been corrected for background activity. (B) Interaction of the P3-derived serum with immobilized immunoaffinity-purified anti-Rev activation domain antibodies. P3 immune-serum preincubated with 20 μg/ml eIF-5A for 30 min at ambient temperature before BIA (P3+eIF-5A; □); P3 immune-serum preincubated with 20 μg/ml BSA for 30 min at ambient temperature before BIA (P3-BSA; □).

Taken together, these data suggest that the reactivity of the P3 antiserum to eIF-5A is an anti-idiotypic response to the initial serum antibodies generated against the Rev activation domain. Thus, the activation domain is the interaction site of the HIV-1 Rev trans-activator with eIF-5A.

Nuclear Localization of eIF-5A

To demonstrate directly that a significant fraction of eIF-5A localizes to the nucleus, we performed indirect immunofluorescence studies.

An anti-eIF-5A polyclonal antiserum was raised in rabbits by immunization of the animals with bacterially expressed glutathione S-transferase-eIF-5A fusion protein (see Materials and Methods). The specificity of the antiserum ob-
tained was verified by immunoprecipitation analysis of eIF-5A protein (data not shown).

We next transfected COS cell cultures with the pELF-5A expression plasmid and subjected the fixed cells to eIF-5A specific indirect immunofluorescence microscopy. No eIF-5A protein was detected upon incubation with pre-immune serum (Fig. 6 A). Using the anti-eIF-5A antiserum, strong cytoplasmic and nuclear fluorescence was easily detectable in transfected cells (Fig. 6 B). In this experiment the exposure time was very short to visualize eIF-5A in overexpressing cells. However, a weak fluorescence originating from endogenous eIF-5A protein could be detected in all cells using a longer exposure time combined with a higher magnification (Fig. 6 C). Clearly, the cytoplasmic fluorescence was faint compared with the more intense signals detectable in the cell nuclei.

To demonstrate the nuclear localization of eIF-5A more clearly, confocal laser scanning microscopy was performed on nontransfected wild-type COS cells (Fig. 7). The fixed cells were incubated together with anti-eIF-5A antiserum and a mouse mAb (CHON 211) that specifically recognizes components of the nuclear pore complex (Park et al., 1987). The immunostaining of the COS cell nuclear envelope by CHON 211 is shown in Fig. 7 A and the eIF-5A specific signal is shown in Fig. 7 B. Combination of both images clearly demonstrated that a significant fraction of eIF-5A is localized to the nucleus (see Fig. 7 C). Similar results were also obtained using HeLa cells (not shown).

Reconstitution of Rev Function in Xenopus Oocytes
by eIF-5A

Having identified eIF-5A as a nuclear binding factor of the HIV-1 Rev activation domain, we next examined whether eIF-5A is also the cellular co-factor required for Rev function. A Northern blot analysis of eIF-5A encoding mRNAs revealed that, in contrast to the cell lines examined (HeLa, CEM, Jurkat, U937), no eIF-5A gene expression was detectable in Xenopus oocytes (see Fig. 8 A). Therefore, the Xenopus oocyte system was chosen to investigate Rev function. An additional Southern blot analysis of the corresponding genomic DNAs using the same eIF-5A-specific probe demonstrated probe specificity (Fig. 8 B). Multiple signals were detected in EcoRI-digested genomic DNA of mammalian cells (Fig. 8 B, lanes 1 to 4). This hybridization pattern might indicate the existence of multiple eIF-5A genes. In contrast, only a single signal was detected in Xenopus DNA (Fig. 8 B, lane 5).

Various Rev expressing plasmids and eIF-5A protein were microinjected into the nuclei of Xenopus oocytes. Rev activity was monitored by coinjection of the Rev-responsive chloramphenicol acetyltransferase (CAT) expression vector pDM128/CMV (Malim et al., 1991). All experiments were internally controlled by addition of the pBC12/CMV/βGal construct. βGal expression was used to equalize the amount of protein extract used in the CAT assay. Before microinjection, the identity and purity of eIF-5A, which was purified as described previously (Park et al., 1986), was confirmed by 2D gel electrophoresis and amino acid sequence analysis.

In these assays Rev failed to induce CAT activity from the Rev-responsive reporter construct pDM128/CMV, indicating the lack of cellular components required for Rev transactivation in oocytes (Fig. 9, lane 3 versus lane 4). However, addition of eIF-5A clearly reconstituted Rev function in the oocytes while eIF-5A alone had no influence on the reporter activity (Fig. 9, lane 5 versus lane 6). In previous studies, mutagenesis of the rev gene combined with functional analysis in mammalian cells was used to generate Rev activation domain mutants with defined phenotypes in vivo. The Rev mutants M10 and M32, both of which have alterations in the
elF-5A Gene Expression Is Required for Rev Trans-Activation in Mammalian Cells

Finally we tested the effect of elF-5A on Rev function in mammalian cells. For this, we transiently blocked elF-5A gene expression in COS cells with an elF-5A antisense construct (peIF-5Aas) and monitored Rev function by co-transfection of the Rev-responsive reporter construct pDM128/CMV. Again, these experiments were internally controlled by inclusion of the pBC12/CMV/βGal expression plasmid in order to rule out an effect of peIF-5Aas on general translation initiation. As summarized in Table II, the expression of elF-5A antisense sequences resulted in a significant inhibition (83%) of Rev function as measured by Rev-dependent CAT activity, indicating the necessity of elF-5A gene expression for Rev function.

Previous studies demonstrated that Rev function directly affects the intracellular distribution of incompletely spliced viral mRNAs in mammalian cells, including COS (Malim et al., 1988, 1989b; Felber et al., 1989). It was shown that HIV-1-derived RRE-containing RNAs are retained in the nucleus and only appear in the cytoplasm when Rev is present. Therefore, we also investigated the effect of peIF-5Aas on the cytoplasmic accumulation of these RNAs. Transfected COS cell cultures were subjected to subcellular fractionation and

Table II. Effect of elF-5A on HIV-1 Rev Activity in Transfected COS Cells

| Transfection* | Level of CAT expression (cpm) | Percent inhibition of Rev function |
|--------------|-------------------------------|----------------------------------|
| pDM128/CMV   | 60                            | -                                |
| pDM128/CMV + pcRev | 15,066                        | -                                |
| pDM128/CMV + pcRev + peIF-5Aas | 2,576                          | 83                               |
| pDM128/CMV + peIF-5Aas | 222                            | -                                |

* 2.5 × 10⁶ COS cells were co-transfected together with 100 ng of pDM128/CMV, 50 ng of pcRev and 500 ng of peIF-5Aas as previously described (Cullen, 1986b). In addition, 60 ng of pBC12/CMV/βGal DNA was included in every transfection. Total input DNA was kept constant by inclusion of the parental expression vector pBC12/CMV as a negative control. Expression of βGal served as internal control in order to normalize the CAT assays.

† Protein extracts were prepared 60 h after transfection and assayed for CAT activity as described previously (Neumann et al., 1987). Values represent the relative levels of CAT expression measured when the change in transacetylation was linear with respect to time and have been corrected for background (mock) activity.
nuclear and cytoplasmic RNAs were subsequently analyzed by SI nuclease protection (see Fig. 10). The probe used in this assay was designed to detect both spliced and unspliced (RRE-containing) RNA species expressed from the Rev-responsive reporter construct pDM128/CMV (Hope et al., 1990; Malim et al., 1991). Attachment of heterologous sequences also allowed us to distinguish the full-length input probe from the fragments rescued by the unspliced and spliced transcripts. As shown in Fig. 10, Rev function resulted in the cytoplasmic accumulation of unspliced RRE-containing RNA (lanes 7 and 2 versus lanes 3 and 4), which is in agreement with previous studies (Malim et al., 1988, 1989b). However, the Rev phenotype, indicated by these cytoplasmic RRE-containing RNAs, was almost completely abrogated by inclusion of the pelF-5A antisense construct (pelF-5Aas) to the transfection (see Fig. 10, lanes 5 and 6). Expression of pelF-5Aas in absence of Rev had no effect on pDM128/CMV expression in a control experiment (Fig. 10, lanes 7 and 8).

The data presented demonstrate that elf-5A gene expression is necessary for Rev function in mammalian cells. elf-5A is critically required for the Rev-mediated cytoplasmic accumulation and expression of incompletely spliced RRE-containing RNAs.

Discussion

The Rev trans-activator protein of HIV-1 plays a key role in the complex regulation of viral gene expression. Rev function results in the expression of the viral structural proteins and hence, in the generation of infectious particles (Terwilliger et al., 1988; Emerman et al., 1989; Hammarskjöld et al., 1989). Experimental data suggested that interaction of Rev with an unknown cellular component is required for Rev function (Malim et al., 1989a, 1991; Mermer et al., 1990; Venkatesh and Chinnadurai, 1990). In this study we demonstrate that eukaryotic initiation factor 5A (elf-5A, previously called elf-4D) is a host co-factor required for Rev trans-activation.

Human elf-5A is a small 154-aa protein with a molecular mass of 16.7 KD (Smit-McBride et al., 1989a). The isoelectric point of the acidic elf-5A was determined previously with a pI of ~5.1, which is in agreement with our results (Park et al., 1986; Smit-McBride et al., 1989b).

elf-5A is the only known cellular protein that contains a hypusine residue, which is formed by post-translational modification of the lysine residue at position 50 within elf-5A (Fig. 4) (Park et al., 1986). The hypusinated protein stimulates the formation of the dipeptide analogue methionyl-puromycin in an in vitro assay which mimics the formation of the first peptide bond during protein synthesis (Park, 1989; Smit-McBride et al., 1989b; Hershey et al., 1990; Park et al., 1991). Thus, elf-5A appears to function on the level of protein synthesis initiation. However, the precise function of elf-5A in vivo is still unknown. elf-5A might have activities in addition to, or instead of, translation initiation (for a recent review see Park et al., 1993).

Implications for Rev Function

The direct interaction of Rev with elf-5A now provides the basis to significantly improve our understanding of Rev-specific trans-activation at the molecular level. It seems reasonable to assume that the targeting of RRE-containing mRNAs to elf-5A could result in an improved utilization of these viral mRNAs by the translational machinery. Recently, D’Agostino and co-workers provided evidence that the presence of Rev affected not only transport, but also translation of viral mRNAs encoding the structural proteins, by promoting polysomal loading (D’Agostino et al., 1992).

The modification or inactivation of translation initiation factors to repress host and enhance viral mRNA translation is a strategy which is used by various viruses. Adenovirus infection involves viral-mediated dephosphorylation of a component of the cap-binding protein complex elf-4F, while poliovirus inactivates elf-4F by proteolytic degradation of an elf-4F subunit (for a recent review see Thach, 1992). Influenza virus, vesicular stomatitis virus and adenovirus are able to control the efficiency with which different mRNAs are translated by blocking phosphorylation of a subunit of the translation initiation factor elf-2.

The identification of elf-5A as a cellular factor interacting with the Rev activation domain allows the model of Rev function to be refined. Rev activity depends on the presence of cis-acting repressive sequences (CRS) which are located in the HIV-1 structural genes and act to retain the viral mRNAs in the nucleus (Rosen et al., 1988; Cochrane et al., 1991; Maldarelli et al., 1991; Schwartz et al., 1992). Inefficient splice-sites present on the viral mRNAs may delay an otherwise very rapid in vivo splicing reaction (Chang and Sharp, 1989). This delay would allow the specific recognition of the RRE RNA sequence by Rev, followed by Rev-specific mul-

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timer formation, which in turn results in the observed dissociation of spliceosomes on the viral mRNAs; a reaction which occurs independent of the Rev activation domain (Kjems et al., 1991b). Finally, the subsequent interaction of the RRE-Rev complex with eIF-5A may not only induce nucleo/cytoplasmic RNA translocation but may also result in the preferential translation of RRE-containing mRNAs. In this model, although Rev initially acts at a nuclear location by binding the viral RNA, it also provides cytoplasmic functions directly affecting translation via eIF-5A.

A Nuclear Role for eIF-5A

eIF-5A was originally described as an abundant cytoplasmic protein which is transiently attached to ribosomes in the course of initiation of eukaryotic cellular protein synthesis. The major evidence for its function comes from a nonphysiological in vitro reaction; the synthesis of methionyl-puromycin (Park, 1989; Smit-McBride et al., 1989b; Hershey et al., 1990; Park et al., 1991). However, the results presented in this study reveal that successful cross-linking of eIF-5A to the Rev activation domain is only achieved with eIF-5A residing in the nuclear compartment (Fig. 1). In agreement with this finding, our eIF-5A-specific immunofluorescence studies clearly demonstrated that a substantial fraction of this protein exists inside the nucleus. It is conceivable that this material was trapped in the nuclear envelope fraction of our initial fractionation experiment (Fig. 1). Nevertheless, this nuclear eIF-5A may possibly extend activities associated with translation initiation. Intriguingly, a similar nuclear localization characterized by speckles (see Fig. 7) was also recently demonstrated by Lejbkowicz and co-workers for a fraction of eukaryotic initiation factor 4E (eIF-4E), which is the mRNA 5' cap-binding protein (Lejbkowicz et al., 1992). Thus it is evident that the nuclear localization of some initiation factors is a common principle in eukaryotic cells, which may serve functions with respect to targeted nucleo-cytoplasmic mRNA transport and/or interference with the nuclear mRNA splicing machinery.

In recent studies the idea of variant eIF-5A activities, including functions other than protein synthesis, have been suggested. In CHO cells as well as in chick embryo fibroblasts, isoforms of eIF-5A could be detected (Park, 1989; Wolff et al., 1992). In the yeast Saccharomyces cerevisiae, two highly similar genes encoding eIF-5A are expressed differentially in response to aerobic growth conditions (Schnier et al., 1991). Two slightly different genes, termed NeIF-5A1 and NeIF-5A2, were also described in tobacco (Chamot and Kuhlemeier, 1992). While NeIF-5A2 appears to be a housekeeping protein involved in general translation initiation, NeIF-5A1 seems to regulate the light-dependent translation of specific transcripts. Taken together, these data indicate that multiple forms of eIF-5A exist which may serve different functions during posttranscriptional regulation of eukaryotic gene expression.

It is tempting to speculate that the subcellular localization of Rev-binding eIF-5A is of functional significance for the selective translocation of specific viral mRNAs across the nuclear envelope. Indeed, the translocation of poly(A)-rich mRNA requires a protein carrier which is part of a complex system located in the nuclear envelope (Gerace and Burke, 1988). It is conceivable that eIF-5A is part of a RNP which is involved in the translocation of nuclear RNA to the cytoplasm. Intriguingly, nuclear RNP are concentrated in discrete nuclear domains, serving as interconnecting channels that extend through the nuclear interior to the nuclear envelope (Gerace and Burke, 1988). Also, the interaction of eIF-5A with the small preribosomal subunit already in the nuclear compartment may result in co-translocation of the viral mRNA. After transit through the nuclear pores and assembly of translation-competent ribosomes, the RRE-containing RNA would be preferentially used in the process of protein synthesis (Arrigo et al., 1991; Lawrence et al., 1991; D'Agostino et al., 1992).

The finding that eIF-5A is a cellular Rev activation domain binding factor required for Rev trans-activation has several consequences. It should now be possible to dissect the complete pathway of Rev function step by step, starting with the specific RRE binding and finally resulting in the preferential translation of these viral mRNAs. It will also be of interest to examine whether other complex retroviruses exploit the same pathway to regulate their gene expression at a posttranscriptional level. Therapeutic interference with Rev-eIF-5A interaction may provide a new opportunity to block viral replication by inhibiting the Rev-regulated expression of the structural proteins.

Clearly, our findings also demonstrate a new biological activity for eIF-5A. Studies aimed to identify the cellular interaction partners of eIF-5A in the nuclear compartment will generate new insights into mechanisms that are applied in eukaryotic cells in order to translate specific transcripts preferentially.

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