Inhibition of HIV-1 Replication in Lymphocytes by Mutants of the Rev Cofactor eIF-5A

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Eukaryotic initiation factor 5A (eIF-5A) is a cellular cofactor required for the function of the human immunodeficiency virus type–1 (HIV-1) Rev trans-activator protein. The majority of a set of eIF-5A mutants did not support growth of yeast cells having an inactivated genomic copy of eIF-5A, indicating that the introduced mutation eliminated eIF-5A activity. Two nonfunctional mutants, eIF-5AM13 and eIF-5AM14, retained their binding capacity for the HIV-1 Rev response element:Rev complex. Both mutants were constitutively expressed in human T cells. When these T cells were infected with replication-competent HIV-1, virus replication was inhibited. The eIF-5AM13 and eIF-5AM14 proteins blocked Rev trans-activation and Rev-mediated nuclear export.

Eukaryotic initiation factor 5A is essential for cell viability and occurs in all eukaryotic cells as well as in archaebacteria. To date, eIF-5A is the only cellular protein known to contain the unusual amino acid hypusine, a modification that might be required for cell proliferation (*1*). Although the exact role of eIF-5A in eukaryotic cells has yet to be resolved, complete intracellular depletion of eIF-5A does not cause major changes in the rate of protein biosynthesis (*2*). Furthermore, eIF-5A is a cellular cofactor of the HIV-1 Rev regulatory protein (*3*).

Trans-activation by the Rev protein is essential for the expression of viral structural proteins and, thus, for HIV-1 replication (*4*). Because Rev activity mediates the translocation of viral mRNAs from the nucleus to the cytoplasm (*5*), it seemed possible that eIF-5A might interact with a nuclear RNA export system. In the nucleus, Rev binds directly to the incompletely spliced HIV-1 mRNAs through the cis-acting Rev response element (RRE) RNA sequence (*6–9*). The activity of Rev is then mediated by its interaction with one or more cellular effector molecules (*10*).

In *Saccharomyces cerevisiae*, two closely related genes encoding eIF-5A, *HYP1* (*TIF51B*) and *HYP2* (*TIF51A*), are regulated reciprocally by heme and oxygen. Under aerobic growth conditions, expression of the *HYP2* gene product is required for cell viability. Human eIF-5A restores viability in Hyp2p-deficient yeast cells (*11*, *12*).

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To elucidate the function of eIF-5A, we mutagenized a complementary DNA (cDNA) encoding human eIF-5A to generate clustered missense mutants (*13*) and then tested the mutants for in vivo activity by the yeast plasmid shuffle technique (*14*).

Table 1. Summary of eIF-5A mutants and yeast complementation data. The eIF-5A proteins are listed on the left. The mutated amino acid positions in the protein and the amino acid changes introduced are listed in the second and third columns, respectively. The ability of various human eIF-5A mutants to complement the *hyp2* disruption in haploid yeast strain WDH#6-9 (*11*) is indicated in the last column. Complementation (Comp.) was scored by analyzing the spontaneous loss of plasmids under nonselective growth conditions and by 5-fluoroortic acid selection. Both techniques gave identical results; aa, amino acid; wt, wild type; +, complementation; −, no complementation. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

eIF-5A protein	Position (aa)	Mutation (aa)	Comp. of *hyp2* disruption
wt	–	–	+
M1	4,5,6	D,L,D →E,D,L	+
M2	9,10	T,G →D,L	+
M3	15,16,17	S,A,T →A,D,L	+
M4	22,23,24	C,S,A →G,D,L	–
M5	43,44,45	M,S,T →I,D,L	–
M6	46,47,48	S,K,T →L,D,L	–
M7	64,65	F,T →D,L	–
M8	69,70	Y,E →D,L	–
M9	75,76	S,T →D,L	–
M10	98,99,100	Y,L,S →L,D,L	–
M11	104,105,106	D,S,G →Q,D,L	+
M12	126,127	K,Y →D,L	+
M13	135,136	I,T →D,L	–
M14	138,139	L,S, →D,L	–
M15	141,142,143	M,T,E →I,D,L	+
M16	149,150	I,K →D,L	–

Complementation was scored in two ways: by analyzing the spontaneous loss of plasmids under nonselective growth conditions and by 5-fluoroorotic acid selection. The expression of the human gene *EIF5A* in yeast was confirmed by Northern (RNA) blot analysis (*15*). Human wild-type eIF-5A and the mutated eIF-5A variants M1, M2, M3, M11, M12, and M15 were able to complement the *hyp2* disruption (Table 1). In contrast, the strains expressing the eIF-5A mutants (M4 to M10, M13, M14, and M16) did not support yeast cell growth.

In gel retardation assays (*16*), the addition of glutathione-S-transferase (GST)–eIF-5A fusion proteins to RRE:Rev complexes resulted in a complex with decreased mobility (Fig. 1A). This complex of decreased mobility was not detected when RRE RNA was incubated with GST or GST–eIF-5A alone, and it was only observed when Rev concentrations that were consistent with the formation of the primary and secondary Rev-specific complex were used.

The eIF-5A–specific signal was depen-

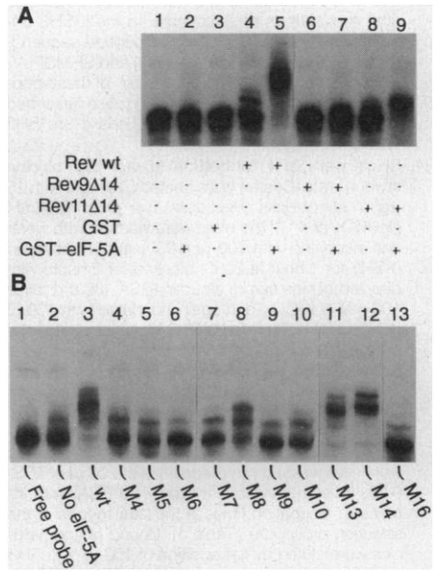

Fig. 1. RNA gel retardation experiments demonstrating interaction of the RRE:Rev nucleic acid: protein complex with GST–eIF-5A. (**A**) Interaction of GST–eIF-5A with the Rev activation domain. (**B**) Binding phenotypes of various GST–eIF-5A mutant proteins (indicated at the bottom) with respect to RRE-bound Rev (lanes 2 through 13). GST, GST–eIF-5A wild-type, and GST–eIF-5A mutant proteins were expressed and purified from *Escherichia coli* and used in conjunction with a constant amount of the indicated Rev proteins (*7*, *17*) and a radiolabeled 252-nucleotide RRE RNA probe. The RNA:protein complexes formed are seen as slower migrating signals after electrophoresis through native polyacrylamide gels followed by autoradiography. The position of unbound RRE is visualized in lane 1 of each gel; wt, wild type.

dent on an activation domain within Rev, as determined with the mutants Rev9Δ14 and Rev11Δ14 (*17*) in our assay. The activation domain is missing in Rev9Δ14 and present in Rev11Δ14. The mutant Rev9Δ14 was incapable of interacting with GST–eIF-5A (Fig. 1A). In contrast, the addition of GST–eIF-5A to the RRE:Rev11Δ14 binding reaction resulted in a new signal (Fig. 1A), indicating interaction of eIF-5A with the Rev activation domain.

Most of the nonfunctional eIF-5A mutant proteins lost their ability to recognize the RRE:Rev complex (Fig. 1B). Only GST–eIF-5AM13 and GST-eIF-5AM14 displayed binding behaviors that were comparable with that of the wild-type protein.

Next, the ability of the eIF-5AM13 and eIF-5AM14 proteins to inhibit HIV-1 replication was tested after retrovirus-mediated gene transfer [with retroviral vector pBC140 (*18*)] into human CEM T cells (*19*). HIV-1 replication in the corresponding CEM cell lines was measured by challenge experiments (*20*) with the replication-competent HIV-1 SF2 strain (*21*). To achieve optimal virus replication, we used titers of HIV-1 [2000 tissue culture infectious doses (TCID)] that were ~40 times those used in studies of trans-dominant Rev protein (*18*) and were comparable with titers typically seen in the peripheral blood mononuclear cells of patients with acquired immunodeficiency syndrome (AIDS) (*22*). In addition, CD4 surface expression was very high (>90%) on all CEM T cell lines tested, allowing effective infection of these target cells by HIV-1.

CEM cells expressing the pBC140 retroviral vector supported replication of HIV-1 SF2 (Fig. 2A). Overexpression of eIF-5A resulted in comparable amounts of p24 Gag in the CEM cell supernatant (Fig. 2A). In sharp contrast, virus replication was inhibited (>93%) in each of three cell cultures constitutively expressing either the eIF-5A mutant M13 or M14 (see Fig. 2, C and D, respectively). As expected, CEM cultures expressing the eIF-5AM9 protein, which was unable to bind the RRE:Rev complex in vitro (Fig. 1), did not display such a marked reduction in virus replication (Fig. 2B).

The low amounts of p24 obtained were not because of a negative effect on cell proliferation. All transduced CEM cell cultures proliferated at similar rates, as demonstrated by the comparable cell numbers (Fig. 2). In addition, de novo infection of wild-type CEM cells could be established with supernatants of all challenged cell lines used in these experiments (*23*), indicating integration of HIV-1. Finally, infectivity studies with recombinant HIV-1 (*24*) in which *nef* was replaced with the *CAT* reporter gene demonstrated that all CEM cell clones investigated were comparably susceptible to virus infection (*23*).

To directly demonstrate that Rev function is indeed inhibited by eIF-5AM13 or eIF-5AM14, we investigated HIV-1 Rev and Tat trans-activation by transient transfection of CEM cell clones expressing the eIF-5A wild-type, M13, or M14 protein (*25*). In agreement with the virus challenge data, Rev trans-activation was blocked in the cells expressing either eIF-5AM13 or eIF-5AM14. In contrast, Tat trans-activation was detectable in all cell lines tested, indicating a specificity of the inhibitory effect for Rev (*26*).

Because the Rev activation domain can act as a nuclear export signal (*27*), we tested whether eIF-5AM13 and eIF-5AM14 also block Rev-mediated nuclear export (Fig. 3). For this experiment, we microinjected GST-Rev fusion proteins into the nuclei of human somatic cells and monitored their nuclear export (*28*). The availability of an automated microinjection system allowed us to routinely microinject ~100 nuclei per experiment, resulting in identical and reproducible data. The GST-RevM32 fusion protein, which is biologically inactive because of a mutation of the protein activation domain (*29*), remained in the nuclei of HeLa cells, whereas GST-Rev wild-type protein was efficiently transported to the cytoplasm.

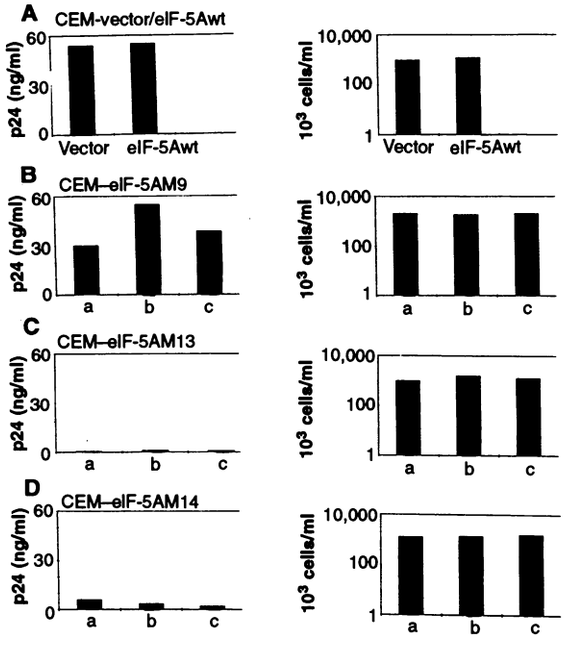

Fig. 2. HIV-1 challenge experiments. (**A** through **D**) Transduced CEM cells were infected for 1 hour at room temperature with concentrated (2000 TCID), replication-competent HIV-1 SF2 and subsequently transferred into fresh medium. After 10 days of incubation, culture medium samples were collected to assay HIV-1 p24 Gag protein by enzyme-linked immunosorbent assay (panels on the left) and to determine cell numbers with the use of a Coulter counter device (panels on the right). Independently obtained clonal populations of transduced CEM cells are indicated by letters a, b, and c.

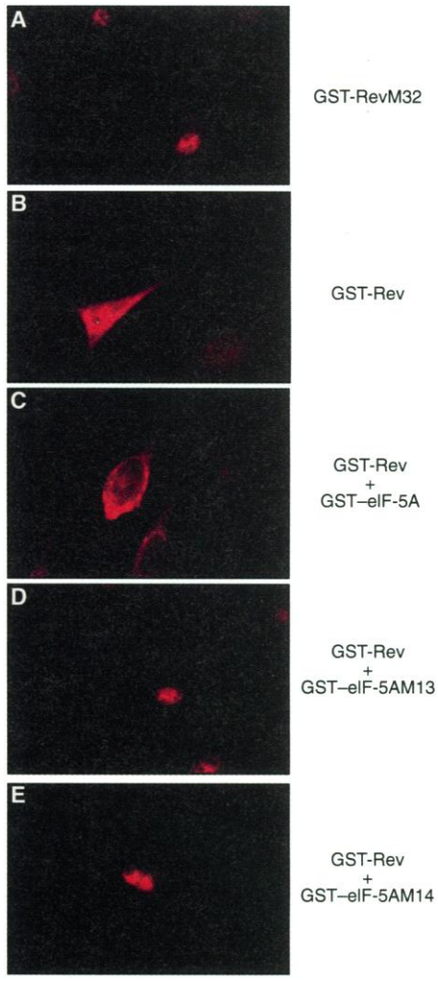

Fig. 3. Inhibition of Rev nuclear export activity by eIF-5AM13 and eIF-5AM14. Nuclei of HeLa cells were microinjected with GST-RevM32 or GST-Rev in combination with bovine serum albumin (BSA) (**A** and **B**) or with a mixture of GST-Rev and the indicated GST–eIF-5A fusion proteins (**C** through **E**). Cells were incubated for ~20 min, fixed, and analyzed for Rev localization by Rev-specific indirect immunofluorescence microscopy.

Coinjection of GST-Rev with GST–eIF-5A resulted in even more efficient Rev export. In sharp contrast, however, coinjection of the GST–eIF-5AM13 or GST–eIF-5AM14 protein completely blocked Rev translocation, directly demonstrating the requirement of eIF-5A for Rev-mediated nuclear export.

The Rev–eIF-5A interaction appears to be a critical site at which the viral parasite connects itself with an important host pathway, thereby providing access to cellular RNA export systems. The phenotypes of the eIF-5A M13 and M14 proteins suggest that they might be useful reagents to be used in gene-based intervention therapies. The constitutive expression of the *EIF5AM13* and *EIF5AM14* genes in human T cells was not toxic, although other cellular proteins (such as protein kinase inhibitor or TFIIIA) share the properties of the Rev activation domain (*27*) and might therefore also be targets of eIF-5A. However, multiple pathways for nuclear export may exist in the cell, and different export complexes could have different affinities for various substrates. The recently published Rev binding protein hRIP (reported also as Rab) (*30*, *31*) might constitute such an alternative export system. The fact that hRIP/Rab cannot be expressed as a recombinant protein (*31*), however, makes it impossible to directly test whether eIF-5A competes with hRIP/Rab for Rev binding. Moreover, a direct interaction of hRIP/Rab with the Rev activation domain has not yet been demonstrated. As discussed in a recent review article (*32*), this interaction may be indirect and could be mediated by a linker protein, such as eIF-5A. It will now be a major goal to identify nuclear interaction partners of eIF-5A. We expect these cofactors to be either components of nuclear-cytoplasmic RNA transport systems or factors that provide access to them.

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13. Oligonucleotide-directed mutagenesis with a bacteriophage M13 mutagenesis system (United States Biochemical, Cleveland, OH) was used to introduce targeted nucleotide substitutions encoding multiple amino acid alterations into the *EIF5A* gene of peIF-5A (*3*). All mutations introduced were confirmed by DNA sequence determination (Sequenase 2.0; United States Biochemical). Protein expression of all mutants was confirmed by eIF-5A–specific immunoprecipitation analysis with radiolabeled protein extracts of transiently transfected COS cells.
14. The yeast *S. cerevisiae* was grown either on semisynthetic medium with 3% galactose or 2% glucose, or on YPD or YPGal media. The complementation assay was performed with a selective or nonselective plasmid shuffle technique, according to Boeke *et al.* [J. D. Boeke, J. Truehart, G. Natsoulis, G. R. Fink, *Methods Enzymol.* **154**, 164 (1987)]. The *hyp2*-disrupted strain WDH#6-9D, containing the wild-type *HYP2* gene borne on the multicopy plasmid YEp-HYP2 (with *URA3* as a selectable marker), served as the haploid host [T. Wöhl, H. Klier, H. Ammer, F. Lottspeich, V. Magdolen, *Mol. Gen. Genet.* **241**, 305 (1993)]. All human *EIF5A* genes were cloned into the plasmid pRSGAL (HIS3 as selectable marker) and transformed independently into strain WDH#6-9D as described (*11*). For a nonselective plasmid shuffle, the resultant strains were grown in YPGal for 5 days with a change of medium every 36 hours. The cells were streaked out on YPGal plates to analyze their retained selectable markers by replica plating. Analysis of spontaneous loss of plasmids was performed in duplicate with at least 100 colonies scored per strain. For selective plasmid shuffle, cells were grown on semisynthetic medium plates containing 0.1% 5-fluoroorotic acid as a selective agent against the *URA3* gene borne on plasmid YEp-HYP2. The absence of the *HYP2* gene product in colonies with a His^+/ura^- phenotype was confirmed by protein immunoblot analysis.
15. D. Bevec, T. Wöhl, J. Hauber, unpublished data.
16. Wild-type and mutant eIF-5A proteins were expressed as COOH-terminal fusions to GST in the *Escherichia coli* strain BL21. The fusion proteins were purified from crude lysates by affinity chromatography on glutathione–Sepharose 4B as described [M. Hammerschmid *et al.*, *J. Virol.* **68**, 7329 (1994)]. Radiolabeled RNA transcripts containing a 252-nucleotide RRE probe were used in the RNA gel retardation assays as described previously (*9*). For gel retardation assays, samples containing 20,000 cpm of radiolabeled RRE RNA were incubated with 0.076 μM Rev protein (*7*, *17*) for 15 min at ambient temperature to allow the formation of RRE:protein complexes (*9*). Subsequently, the GST–eIF-5A proteins (3.2 μM of each mutant) were added and incubation continued for an additional 45 min. All reactions also contained 2 μg of MS-2 RNA (Boehringer Mannheim, Mannheim, Germany) and 5 units RNasin (Promega, Madison, WI) in TBE (89 mM tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.4) buffer. The total sample (volume 12 μl) from each reaction was subjected to electrophoresis for 2 hours at 15 mA with native 5% polyacrylamide gels. Finally, the gels were dried and subjected to autoradiography.
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19. The retroviral vectors were constructed by inserting polymerase chain reaction (PCR)–generated *EIF5A* genes into the single Xho I site of the retroviral vector pBC140 (*18*). The eIF-5A–coding regions of all retroviral constructs generated were determined by DNA sequencing with Sequenase 2.0. Twenty micrograms of each retroviral DNA were electroporated into the amphotropic GP + envAm12 (Am12) packaging cell line [D. Markowitz, S. Goff, A. Bank, *Virology* **167**, 400 (1988)] as described previously [D. Bevec *et al.*, *Hum. Gene Ther.* **5**, 193 (1994)]. $CD4^+$ human CEM cells were inoculated as described (*18*) with virus-containing, infectious Am12 supernatants and selected for neomycin-resistant CEM cell populations. RNA expression of the transgene was characterized in clonal populations of the transduced CEM cells as described (*18*). Vectors expressing the eIF-5A wild-type and the randomly selected eIF-5AM9 gene served as controls in the experiments.
20. Clonal populations of 2×10^6 CEM T cells were infected with high doses (2000 TCID; determined by end-point titration) of HIV-1 SF2 virus at ambient temperature for 1 hour. Subsequently, the cells were diluted in fresh RPMI 1640 medium containing 10% fetal bovine serum and incubated at 37°C. After 10 days, samples of the culture medium were collected and assayed for HIV-1 replication, as measured by p24 Gag protein synthesis, with an antigen capture assay (enzyme-linked immunosorbent assay). In parallel, cell counts were determined with a Coulter device.
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25. CEM T cells (10^7) were transiently transfected with 3 μg of the Rev reporter pDM128/CMV (*29*) or the Tat reporter pBC12/HIV/CAT [J. Berger, J. Hauber, R. Hauber, R. Geiger, B. R. Cullen, *Gene* **66**, 1 (1988)] and 3 μg of the parental vector pBC12/CMV (negative control), pcREV, or pcTAT [M. H. Malim, J. Hauber, R. Fenrick, B. R. Cullen, *Nature* **335**, 181 (1988)] with DEAE-dextran and chloroquine. In addition, 3 μg of pBC12/CMV/βGal DNA (*3*) was included in every transfection. Protein extracts were prepared 48 hours after transfection and assayed for β-galactosidase and chloramphenicol acetyltransferase activity.
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28. HeLa cells were microinjected in the nucleus with a Zeiss Axiovert inverse microscope and a Zeiss automated injection system AIS (Zeiss, Oberkochen, Germany) in combination with an Eppendorf microinjector 5242 (Eppendorf Gerätebau, Hamburg, Germany). The GST-Rev and GST-RevM32 proteins were injected at a concentration of 0.5 mg/ml, together with the respective GST–eIF-5A isoform or BSA (1.25 mg/ml). The injection procedure was carried out as described previously [W. Ansorge, *Exp. Cell Res.* **140**, 31 (1982)]. About 20 min after injection, cells were fixed with 3% paraformaldehyde, 1% BSA in phosphate-buffered saline for 10 min, triton-treated for 10 min, and subsequently stained, as described previously, with a mouse monoclonal antibody to Rev [M. Hammerschmid *et al.*, *J. Virol.* **68**, 7329 (1994)]. Immunofluorescence stainings were analyzed with a Zeiss axiophot microscope with a KAF 1400 CCD camera (Photometrics, Tuscon, AZ) and IPLab spectrum and Adobe Photoshop 3.0 software.
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