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Induced gene expression of the hypusine-containing protein eukaryotic initiation factor 5A in activated human T lymphocytes

(T-cell activation/T-cell antigen receptor/primary human lymphocytes/human immunodeficiency virus Rev protein)

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ABSTRACT **The hypusine-containing protein eukaryotic initiation factor 5A (eIF-5A) is a cellular cofactor critically required for the function of the Rev transactivator protein of human immunodeficiency virus type 1 (HIV-1). eIF-5A localizes in the nuclear and cytoplasmic compartments of mammalian cells, suggesting possible activities on the level of regulated mRNA transport and/or protein translation. In this report we show that eIF-5A gene expression is constitutively low but inducible with T-lymphocyte-specific stimuli in human peripheral blood mononuclear cells (PBMCs) of healthy individuals. In contrast, eIF-5A is constitutively expressed at high levels in human cell lines as well as in various human organs. Comparison of eIF-5A levels in the PBMCs of uninfected and HIV-1-infected donors shows a significant upregulation of eIF-5A gene expression in the PBMCs of HIV-1 patients, compatible with a possible role of eIF-5A in HIV-1 replication during T-cell activation.**

Eukaryotic initiation factor 5A (eIF-5A) is the only cellular protein known to contain the unusual amino acid hypusine. The hypusine modification is formed posttranslationally by the transfer of an aminobutyl moiety from spermidine to a specific lysine residue in the 16.7-kDa eIF-5A protein which is subsequently hydroxylated (reviewed in ref. 1). The hypusine formation appears to be essential for cell proliferation (2, 3). The notion that eIF-5A is involved in protein translation initiation comes from its ability to stimulate the formation of the dipeptide analogue methionylpuromycin in an *in vitro* assay (4–8). However, further data have suggested that the observed *in vitro* activity of eIF-5A may be an artifact of the assay system used (9). This would explain the reported failure to demonstrate the stimulation of globin mRNA translation by eIF-5A (10).

The idea that eIF-5A fulfills *in vivo* functions other than an activity in overall translation initiation was also supported by a recent study using the yeast *Saccharomyces cerevisiae*. In this work the authors demonstrated that protein synthesis continued well after complete intracellular depletion of eIF-5A (11). The indication of a potentially new eIF-5A *in vivo* activity emerges from a recent report showing that eIF-5A is a cellular cofactor of the human immunodeficiency virus type 1 (HIV-1) Rev transactivator protein (12). These studies revealed that a significant fraction of eIF-5A accumulated in mammalian cell nuclei, a localization which might be due to a function other than in protein translation initiation. It was also shown that eIF-5A gene expression was required in mammalian cells for the Rev-mediated nucleo/cytoplasmic translocation of HIV-1-specific mRNAs. These data indicated that nuclear eIF-5A might be part of a cellular pathway involved in the transport of nuclear mRNA to the cytoplasm (12). In fact, unexpected dual nuclear and cytoplasmic functions have been reported for a series of RNA-binding proteins (13), including the mRNA 5′-cap-binding protein eIF-4E (14).

Here we investigate the level of eIF-5A gene expression in various mammalian cells and tissues and in HIV-infected individuals. Our data reveal that eIF-5A is significantly inducible in primary human peripheral blood mononuclear cells (PBMCs) by physiological stimuli, indicating a role of eIF-5A in the immune response and/or cell proliferation.

MATERIALS AND METHODS

Cells. Heparinized human peripheral blood was collected from healthy adult volunteers or HIV-1-infected patients after informed consent was given. PBMCs were obtained by standard techniques (Lymphoprep; Nycomed, Oslo). T lymphocytes from healthy donors were separated from the heterogeneous mononuclear cells by rosetting them with neuraminidase-treated sheep erythrocytes. Subsequently, these cells were cultured in a 5% CO_2 atmosphere at 37°C in complete RPMI 1640 medium supplemented with either 10% fetal bovine serum or 10% autologous donor plasma. PBMCs from HIV-1-serpositive men were staged according to the Centers for Disease Control and Prevention (CDC; Atlanta) classification as follows: patient A, CDC IIIB (A3), 80 $CD4^+$ cells per mm^3; patient B, CDC IVC1 (C3), 50 $CD4^+$ cells per mm^3, cerebral toxoplasmosis; patient C, CDC IIB (A2), 360 $CD4^+$ cells per mm^3; patient D, CDC IIB (A2), 300 $CD4^+$ cells per mm^3.

The cell lines examined in this study included the human T-cell line CEM, the human monocytic line Mono-Mac 6, and various cell lines from different species: NIH 3T3 (mouse), COS-1 (African green monkey), MDCK (dog), RK13 (rabbit), NRK (rat), CHO (hamster), and LLCMK2 (rhesus monkey).

Stimulation with Antibodies. Tissue culture plates (35 mm; Costar) were coated overnight at 4°C with goat anti-mouse immunoglobulin G1 (10 μg/ml; Southern Biotechnology Associates). In these plates human PBMCs or isolated T cells from healthy donors were incubated in the presence of various immobilized monoclonal antibodies (mAbs): anti-CD3 (OKT3) and anti-CD4 (OKT4; both from Ortho Diagnostics); anti-CD2 (OKT11; American Type Culture Collection; and VIT 13; Institute of Immunology, University of

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Abbreviations: eIF-5A, eukaryotic initiation factor 5A; HIV-1, human immunodeficiency virus type 1; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; TCR, T-cell antigen receptor; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; SEA, *Staphylococcus* enterotoxin A; IL, interleukin.

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Vienna); anti-CD8 (DK25), anti-HLA class I antigen (W6/32), anti-HLA-DR (DK22), and anti-HLA-DRα (TAL.1B5; all from Dakopatts, Glostrup, Denmark); anti-T-cell antigen receptor (TCR) α/β (BL-A8), anti-β_2-microglobulin (B2M-01), and anti-CD71 (MEM75; all from Sanbio, Uden, The Netherlands); anti-actin (C4) and anti-HLA-DRβ (CR3/43; both from Boehringer Mannheim); and anti-CD28 (CLB-402; Janssen). Control stimulations were performed by exposure of the cells to phytohemagglutinin (PHA, 1 μg/ml) plus phorbol 12-myristate 13-acetate (PMA, 50 ng/ml), ionomycin (200 ng/ml), bacterial lipopolysaccharide (LPS, 1–10 μg/ml; from *Escherichia coli* 055; B5; Sigma), *Staphylococcus* enterotoxin A (SEA, superantigen, 10 μg/ml), or interleukin 2 (IL-2, 10–200 units/ml). After various periods of incubation, the cells were analyzed for mRNA expression and eIF-5A synthesis.

Northern Blot Analysis. Total cellular RNA was isolated from cultured cells and human tonsils (15). Commercially purchased Northern blot membranes (Clontech) were used to detect specific mRNAs in human organs other than tonsils and PBMCs. For hybridization, α-^{32}P-radiolabeled oligonucleotide probes were used (16). The following specific oligonucleotide probes were designed for mRNA detection: eIF-5A (nt 301–330; ref. 17), 5′-CCT-GGA-TGC-CAA-TCA-GCT-GGA-AGT-CAT-TCC-3′; IL-8 (nt 202–228; ref. 18), 5′-GGG-GTG-GAA-AGG-TTT-GGA-GTA-TGT-CTT-3′; β-actin (nt 401–434; ref. 19), 5′-GGC-TGG-GGT-GTT-GAA-GGT-CTC-AAA-CAT-GAT-CTG-G-3′.

Protein Detection. PBMCs of healthy donors were incubated with immobilized anti-CD3 mAbs or control medium for 48 hr. Then the cells were lysed in 9 M urea/1% (vol/vol) Nonidet P-40/5% (vol/vol) 2-mercaptoethanol/0.4% (wt/vol) Ampholine carrier ampholytes, pH 3.5–10.0/0.4% (wt/vol) Pharmalytes, pH 3.5–10.0 (Pharmacia) and protein samples were subjected to two-dimensional gel electrophoresis. Isoelectric focusing (20) was carried out with immobilized-pH-gradient gel strips (IPG Dry Plates; Pharmacia) at 15°C using paraffin protection. During the first 2 hr, the samples were focused with 300 V. Every 2 hr the voltage was increased by 300 V, up to 1200 V. During the last hour the focusing was run at 4000 V to reach a total value of 40 kV·hr. The gel strips were equilibrated in 6 M urea/2% (wt/vol) sodium dodecyl sulfate (SDS)/1% (wt/vol) dithiothreitol/30% (vol/vol) glycerol/50 mM Tris, pH 6.8/0.8 mM EDTA at ambient temperature.

For SDS/polyacrylamide gel electrophoresis (PAGE) in the second dimension, the equilibrated gel strips were placed on top of Laemmli gels (21). After electrophoretic separation, the two-dimensional gels were blotted onto Fluorotrans membranes (Pall) as described (22). Western blot analysis was performed with polyclonal rabbit anti-eIF-5A antibodies (12).

RESULTS

Constitutive eIF-5A Gene Expression in Various Species. eIF-5A has been considered to be an abundant protein that is constitutively expressed in accordance with its supposed activity in overall protein translation initiation (1). To test this assumption, we first subjected various RNAs to Northern blot analysis. An eIF-5A-specific signal was easily visualized in the RNA from all cell lines tested (Fig. 1, lanes 1–9). Surprisingly, only low levels of eIF-5A mRNA were detectable in unstimulated PBMCs derived from healthy human donors (Fig. 1, lane 10). Therefore, we extended our analysis to RNAs extracted from human organs and PBMCs. Clearly, eIF-5A mRNA is expressed at elevated levels in differentiated human tissue when compared with PBMC-derived RNA (Fig. 2, lane 1 versus lanes 2–12).

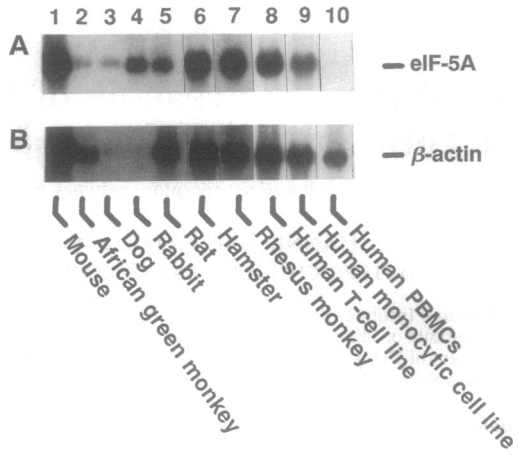

FIG. 1. eIF-5A-specific RNA analyses of cell lines from various species and unstimulated primary human PBMCs. (*A*) Samples (10 μg) of total RNA (15) from mouse (NIH 3T3, lane 1), African green monkey (COS-1, lane 2), dog (MDCK, lane 3), rabbit (RK 13, lane 4), rat (NRK, lane 5), hamster (CHO, lane 6), and rhesus monkey (LLC MK2, lane 7) cells, human T-lymphocyte line CEM (lane 8), human monocytic line Mono-Mac-6 (lane 9), and primary human PBMCs (lane 10) were subjected to Northern blot analysis. Filters were hybridized with a ^{32}P-labeled eIF-5A-specific oligonucleotide probe. (*B*) Radioactivity was washed off the membranes used in *A* and rehybridized with a β-actin-specific oligonucleotide probe for control of RNA loading.

eIF-5A Gene Expression Is Inducible in Human PBMCs. The following experiments were designed to elucidate whether eIF-5A gene expression could be induced in unstimulated human PBMCs (Figs. 3 and 4). PBMCs of healthy individuals were incubated with a set of activating agents, including general stimulators of blood cells such as PHA/PMA, ionomycin, LPS, IL-2, or bacterial superantigen (SEA). Furthermore, mAbs directed against specific molecules on the surface of leukocytes (TCR α/β, CD2, CD3, CD4, CD8, or CD28) were used. In control experiments, cells were also exposed to immobilized antibodies directed against β-actin, CD71, HLA class I, HLA class II, or β_2-microglobulin, which are common to the majority of blood cells. To provide an activity control for the inducing agents employed, gene induction for IL-8-specific mRNA was also monitored

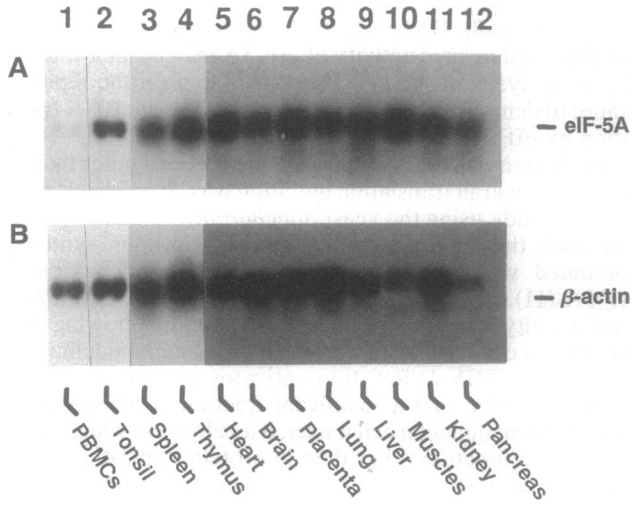

FIG. 2. Northern blot analysis of eIF-5A-specific transcripts in various human organs. Twenty micrograms of total RNA was loaded on lane 1 (unstimulated PBMCs) and lane 2 (tonsil). Lanes 3–12 show 2 μg of mRNA from the indicated organs on filters purchased from Clontech. (*B*) Detection of β-actin mRNA after rehybridization.

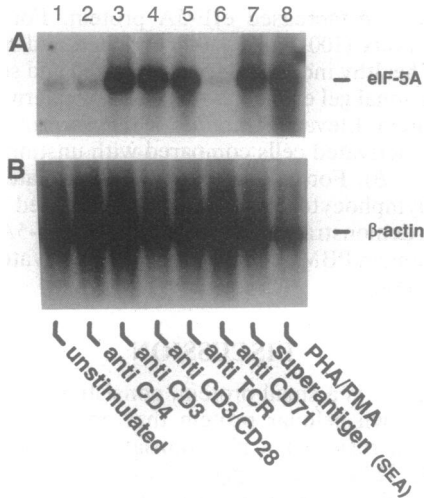

FIG. 3. Induction of eIF-5A gene expression in healthy donors' PBMCs. Sample (10 μg) of total RNA was hybridized to a ^{32}P-labeled eIF-5A-specific oligonucleotide (*A*) or to a ^{32}P-labeled β-actin probe (*B*). Lane 1, RNA from unstimulated PBMCs; lanes 2–8, RNA from PBMCs stimulated with anti-CD4 (lane 2), anti-CD3 (lane 3), anti-CD3 plus anti-CD28 (lane 4), anti-TCRα/β (lane 5), anti-CD71 (lane 6), SEA (lane 7), or PHA/PMA (lane 8).

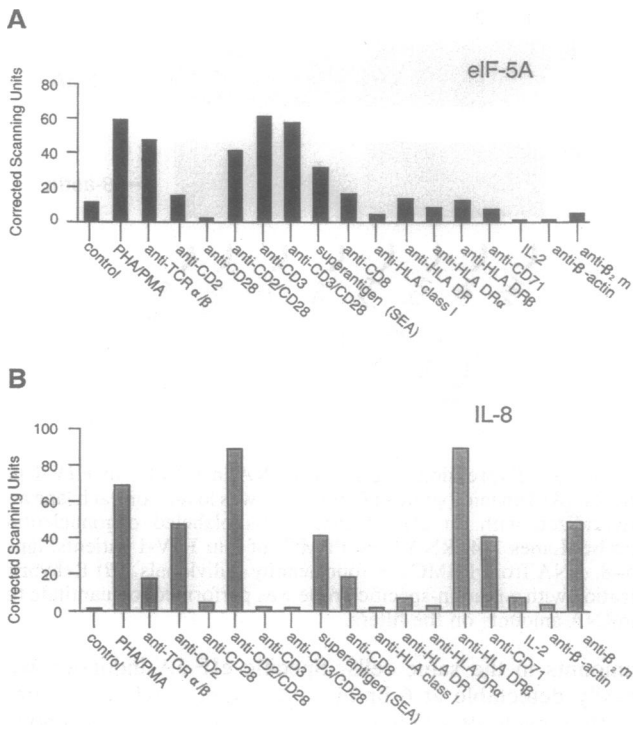

FIG. 4. Comparison of eIF-5A gene induction (*A*) and IL-8 gene induction (*B*) in PBMCs from a healthy donor after 48 hr of stimulation with various inducing agents. Total RNA (10 μg per sample) was subjected to Northern blot analysis using eIF-5A-, IL-8-, and β-actin-specific oligonucleotide probes. Bars indicate the relative gene induction as determined by densitometric analysis and normalization to the β-actin control gene. β_2m, β_2-Microglobulin.

(23, 24) (Fig. 4*B*). Detection of IL-8 mRNA accumulation was chosen because the IL-8 gene is easily inducible, maintaining high and long-lasting mRNA levels upon stimulation with anti-CD2/CD28 antibodies, allowing direct comparison of the gene induction profiles for IL-8 and eIF-5A even 48 hr after stimulation begins. This stimulation was also reported to result in a prolonged accumulation of other cytokine mRNAs (25). Interestingly, in our experiments the IL-8 mRNA level was not elevated by anti-CD3 or anti-CD3/CD28 antibodies at 48 hr (Fig. 4*B*), although it was easily detectable earlier (at 24 hr) (D.B. and W.H., unpublished results), indicating that optimal concentrations of stimuli were used. mRNA accumulation was visualized by Northern blot analysis.

eIF-5A gene induction was easily achieved upon stimulation with the combination of PHA and PMA (Fig. 3, lane 8) and with the more T-cell-specific triggers anti-CD3 mAb, anti-TCRα/β mAb, a combination of anti-CD3 and anti-CD28 mAbs, and also with superantigen SEA (Fig. 3, lanes 3–5 and lane 7, respectively). Furthermore, costimulation with anti-CD2 and anti-CD28 mAbs (Fig. 4*A*), as well as stimulation using ionomycin (data not shown), displayed a clear effect on eIF-5A gene expression. No significant stimulatory effect was observed upon induction with anti-CD4 mAb or anti-CD71 mAb (Fig. 3, lanes 2 and 6) or with anti-CD2, anti-CD8, anti-CD28, anti-HLA class I, anti-HLA-DR, anti-β-actin, or anti-β_2-microglobulin mAb or IL-2 (Fig. 4*A*). Of note was the result that, while LPS or various muramyl peptides had no effect on eIF-5A mRNA levels, they did induce genes encoding IL-8, IL-1, tumor necrosis factor α, or monocyte chemotactic and activating factor, indicating specificity of the observed gene activation (data not shown). These data demonstrate that the eIF-5A gene can be induced significantly in human PBMCs by T-lymphocyte-stimulating agents. In addition, treatment of the cells before or during the stimulation procedure with cyclosporin A, a powerful immunosuppressive drug (26), did not inhibit either PHA/PMA- or anti-CD3-induced eIF-5A mRNA accumulation (data not shown).

Kinetics of eIF-5A Gene Induction. Isolated T lymphocytes of healthy donors were costimulated either with PHA/PMA or with anti-CD3 and anti-CD28 mAbs for up to 72 hr as described above. At certain time points the cells were analyzed for eIF-5A mRNA (Table 1). The specificity of eIF-5A induction was measured relative to the β-actin mRNA

Table 1. Kinetics of the specific eIF-5A gene induction in healthy donors' PBMCs upon stimulation either with PHA/PMA or with anti-CD3/CD28 antibodies

Time of induction, hr	PHA/PMA		Anti-CD3/CD28	
	Densitometer units	Fold eIF-5A induction	Densitometer units	Fold eIF-5A induction
0	12.23	1	12.23	1
6	99.27	8.1	221.85	18.1
12	496.90	58.4	329.20	38.7
24	612.40	28.8	641.80	30.2
48	192.10	14.9	168.90	13.1
72	9.00	1	11.00	1

T lymphocytes of healthy individuals were costimulated either with PHA and PMA or with anti-CD3 and anti-CD28 mAbs as described in *Materials and Methods*. At the indicated times the cells were analyzed for eIF-5A mRNA levels by Northern blot analysis using laser densitometry for quantification.

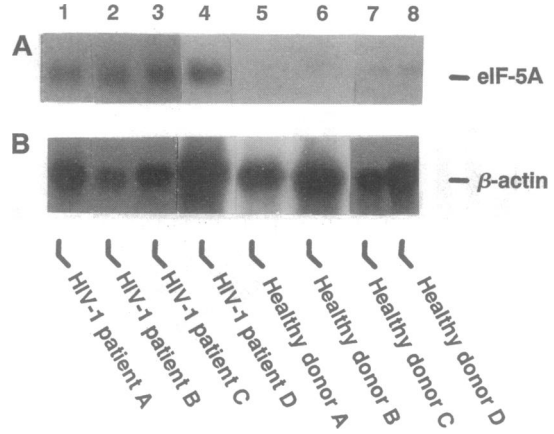

FIG. 5. Expression of eIF-5A mRNA in PBMCs of HIV-1 patients. (*A*) Ten micrograms of total RNA was loaded on each lane and hybridized with an eIF-5A-specific radiolabeled oligonucleotide probe. Lanes 1–4, RNA from PBMCs of four HIV-1 patients; lanes 5–8, RNA from PBMCs of four healthy individuals. (*B*) Rehybridization with a β-actin-specific probe was performed to quantitate the mRNA amounts on the filters.

amounts in the same cells. Specific eIF-5A induction was easily detectable at 6 hr of stimulation, reaching maximal mRNA levels after 12 hr of induction. After 72 hr, the amount of eIF-5A mRNA declined to the initial basal level (Table 1). These results demonstrate that activation of primary human T lymphocytes via the TCR/CD3 complex transiently elevates eIF-5A gene expression to mRNA levels comparable to those seen in cell lines and human organs.

eIF-5A mRNA Levels in PBMCs of HIV-1 Patients. Recent work has demonstrated that eIF-5A is a cellular binding factor of the Rev protein of HIV-1 (12). Furthermore, eIF-5A gene expression is critically required for Rev function, resulting in the expression of the viral structural proteins. Therefore, we compared the eIF-5A-specific mRNA levels in PBMCs of healthy individuals with the levels in patients infected with HIV-1, having $CD4^+$ cell counts between 50 and 360 per mm^3. A representative Northern blot analysis is shown in Fig. 5. In total, the PBMCs of 20 healthy donors and 10 HIV-1 patients were subjected to this analysis and yielded comparable results. Consistent with Figs. 1–3, eIF-5A mRNA was almost undetectable in the PBMCs of healthy volunteers (Fig. 5, lanes 5–8). In contrast, high levels of eIF-5A transcripts were detected in the PBMCs of HIV-1 patients (Fig. 5, lanes 1–4). These results suggest strong T-cell activation in HIV-1 patients' PBMCs, which in turn may facilitate HIV-1 Rev function in those cells by induction of eIF-5A.

Increased eIF-5A Protein in Activated Human PBMCs. Having demonstrated induced eIF-5A mRNA levels due to T-cell activation in human PBMCs, we examined whether these mRNAs were also utilized for protein synthesis, which should result in increased eIF-5A protein. For this, total protein extracts (100 μg) of 48-hr-induced and noninduced PBMCs of healthy individuals were isolated and subjected to two-dimensional gel electrophoresis and Western analysis of eIF-5A protein. Elevated amounts of eIF-5A protein could be detected in activated cells compared with unstimulated cells (Fig. 6 *C* vs. *B*). For comparison, eIF-5A isolated from the human T-lymphocyte line CEM was analyzed (Fig. 6*A*). These data demonstrate that the increase in eIF-5A mRNA in activated human PBMCs directly results in elevated levels of eIF-5A protein.

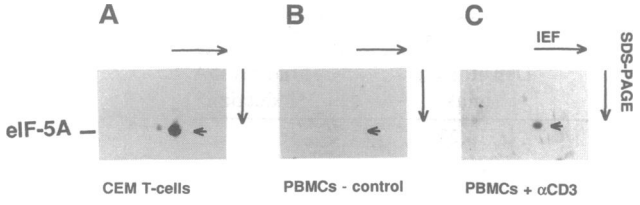

FIG. 6. eIF-5A is increased in activated PBMCs from healthy volunteers. Western blot analysis of two-dimensional gels of eIF-5A protein from the T-cell line CEM (*A*) or from unstimulated (*B*) or anti-CD3 stimulated (*C*) PBMCs is shown. Membranes were stained with an anti-eIF-5A antibody (12). Small arrows indicate eIF-5A protein. IEF, isoelectric focusing.

DISCUSSION

Human eIF-5A is a small protein, consisting of 154 aa. It is the only known cellular protein that contains a hypusine residue, as a result of a posttranslational modification of Lys^{50} (1, 2). It has been suggested that eIF-5A functions as an initiation factor of overall protein synthesis. However, data leading to this hypothesis were obtained from a nonphysiological *in vitro* assay (5–8) and remain controversial (9). Nevertheless, the hypusine modification in eIF-5A seems to be crucial for cellular proliferation (2, 3), although the precise *in vivo* function of eIF-5A is unknown (1). Recently the direct interaction of the HIV-1 Rev transactivator protein with eIF-5A was shown to be essential for Rev transactivation (12). In addition, a significant fraction of eIF-5A appears to accumulate in the nuclear compartment in mammalian cells, a subcellular localization that may be of functional significance for eIF-5A *in vivo* activity. It was suggested that eIF-5A regulates the selective transport of specific mRNAs across the nuclear envelope, resulting indirectly in increased protein translation. In the case of HIV-1 infection, this cellular pathway appears to be exploited by the Rev protein in order to express the viral structural proteins (27).

In the present study we show that eIF-5A gene expression is highly elevated in human cell lines as well as in human organs (Figs. 1 and 2). In contrast, only a very low level of eIF-5A steady-state mRNA accumulation can be detected in resting PBMCs of healthy donors (Figs. 3 and 4). To investigate whether eIF-5A gene expression can be induced in these cells, we have incubated PBMCs of healthy volunteers with various stimulating agents. The high levels of eIF-5A gene expression that were observed in various cell lines (Fig. 1) and organs (Fig. 2) could be achieved in healthy donors' PBMCs only upon stimulation with PHA/PMA, bacterial superantigen, anti-CD3 mAb, anti-TCR α/β mAb, or combinations of anti-CD2 and anti-CD28 or anti-CD3 and anti-CD28 mAbs (Figs. 3 and 4) or with ionomycin (data not shown). These data indicate that the specific upregulation of eIF-5A gene expression is mediated by stimuli of T-cell activation and/or proliferation.

The observed induction of the eIF-5A gene is a transient cellular effect (Table 1). Maximal eIF-5A mRNA levels were obtained 12 hr after stimulation with PHA/PMA or anti-CD3 mAb. After 3 days of stimulation, eIF-5A mRNA levels dropped to the initial basal values (Table 1). The increase in eIF-5A mRNA resulted in increased synthesis of eIF-5A protein (Fig. 6), which is in agreement with the previous report on hypusine formation in proliferating lymphocytes (3). Interestingly, the treatment of PBMCs with cyclosporin A, a powerful immunosupressive drug that inhibits lymphokine synthesis in T lymphocytes at the level of gene transcription (26, 28, 29), did not suppress either anti-CD3- or PHA/PMA-induced eIF-5A mRNA accumulation (data not shown). These data suggest a different mechanism of regulation of gene induction for eIF-5A compared with that of typical T-cell markers such as IL-2 or IL-2 receptor (30, 31). Of note, LPS and muramyl peptides, which act as powerful cytokine inducers in monocytes, had no effect on eIF-5A

gene expression even at high concentrations (up to 10 μg/ml; data not shown). In contrast, the human monocytic cell lines Mono-Mac-6 (Fig. 1) and U-937 (32) show strong constitutive eIF-5A gene expression.

Induction of HIV-1 replication in primary human T lymphocytes requires cell activation (33). T-cell-stimulating agents such as PHA, PMA, anti-TCR/CD3 complex antibodies, or anti-CD28 mAbs also trigger initial HIV-1 gene transcription by activating transcription factors that bind to enhancer sequences located in the HIV-1 long terminal repeat promoter (34). Furthermore, expression of the HIV-1 structural proteins requires the presence of functional viral Rev protein (12, 27, 35). Our data suggest that T-cell-specific signals not only initiate HIV-1 gene transcription from the HIV-1 promoter but also provide increased levels of the Rev cofactor eIF-5A by stimulating eIF-5A gene expression. In contrast to primary cells, the human T-cell lines CEM (Figs. 1 and 6) and Jurkat (data not shown) express eIF-5A mRNA at high constitutive levels. Both cell lines strongly support HIV-1 replication without further cellular stimulation. Therefore, it is tempting to speculate that strong eIF-5A expression is one possible factor that contributes to efficient HIV-1 replication in these cells. Consistent with this model system, a similar pattern of expression was also observed in PBMCs of HIV-1 patients (Fig. 5), where all tested individuals had significantly elevated eIF-5A mRNA levels. It is not clear whether these eIF-5A mRNA levels were due to an additive response to multiple antigens over time or due to polyclonal stimulation of the T-cell population in infected individuals.

In addition to its role as cofactor of the HIV-1 Rev protein, involved in the nucleo/cytoplasmic translocation of viral mRNAs, eIF-5A might perform similar functions in the cellular context. For example, eIF-5A may be involved in the transport of specific mRNAs which encode proteins required for effective T-cell immune response or T-cell proliferation.

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