RESEARCH ARTICLE

Modifications in the human T cell proteome induced by intracellular HIV-1 Tat protein expression

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The effects of the human immunodeficiency virus type 1 (HIV-1) Tat protein on cellular gene expression were analysed using a Jurkat cell line that was stably transfected with *tat* gene in a doxycycline-repressible expression system. Expressed Tat protein (aa 1–101) was proved to present basically a nuclear localisation, and to be fully functional to induce HIV LTR transactivation. Tat expression also resulted in protection from Tunicamycin-induced apoptosis as determined by DNA staining and TUNEL assays. We applied proteomics methods to investigate changes in differential protein expression in the transfected Jurkat-Tat cells. Protein identification was performed using 2-D DIGE followed by MS analysis. We identified the down-regulation of several cytoskeletal proteins such as actin, β-tubulin, annexin II, as well as gelsolin, cofilin and the Rac/Rho-GDI complex. Down-expression of these proteins could be involved in the survival of long-term reservoirs of HIV-infected CD_4^+ T cells responsible for continuous viral production. In conclusion, in addition to its role in viral mRNA elongation, the proteomic approach has provided insight into the way that Tat modifies host cell gene expression.

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1 Introduction

Human immunodeficiency virus type 1 (HIV-1) establishes long-term infection of a certain population of CD_4^+ host cells, which could become long-lived reservoirs for this virus. Persistent infection is characterised by a gradual decrease in CD_4^+ T cell number, ultimately leading to AIDS. Together with a direct viral cytopathic effect, it has been suggested that apoptosis is an important mechanism in CD_4^+ T cell depletion. Apoptosis seems to be induced mainly in non-infected cells through interaction of HIV receptors with viral particles, envelope proteins [1], and extracellular Tat [2]. Surprisingly, infected T cells are relatively protected from apoptosis [3, 4]. A possible explanation for the survival of HIV-infected T cells is that viral infection could modify genetic cell programs inducing an apoptosis-resistant phenotype [4].

HIV-1 transactivating protein (Tat) is a prerequisite for productive viral replication. Tat is a potent activator of viral gene expression and replication [5] and regulates the expression of a variety of genes, including DNA-binding proteins, receptors, and membrane proteins [6]. Tat also has a remarkable property: it can leave cells from which it is synthesised and cross the membrane of adjacent cells, where it localises in the nucleus, transactivates a number of genes, and modulates certain cellular activities on its own [7–10].

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Abbreviations: EGFP, enhanced green fluorescent protein; **HIV-1**, human immunodeficiency virus type 1; **LTR**, long terminal repeat

Although a great variety of gene expression changes have been described in different cell types by HIV infection, in the present study we analysed the effects of constitutive intracellular Tat expression in a lymphoid T cell. To this aim, a Jurkat cell line was stably transfected using a doxycycline-repressible (Tet-Off) expression system. A strong, constitutive expression of viral Tat protein is obtained in these cells and the addition of doxycycline to the culture medium can turn it off. This mechanism assures the continuous maintenance of Tat expression in these cells and avoids cell toxicity caused by Tat expression. A high percentage of the cell population was constitutively expressing Tat with capacity to transactivate regulatory TAR elements in the HIV long terminal repeat (LTR) promoter. Surprisingly, although Tat toxicity and apoptotic capacity have been described previously, in our cell model Tat expression resulted in protection from apoptosis.

On the other hand, changes in the proteome of Jurkat-Tat cells were analysed to determine how Tat expression can influence the levels of certain proteins. The analyses performed with DIGE followed by MS revealed a down-regulation of proteins involved in cytoskeletal activities in Tat-expressing cells. Because some viral proteins interact with cytoskeletal proteins causing profound cytoskeletal reorganisations that can finally induce apoptosis, the down-expression of these proteins could control these cytoskeletal changes and therefore maintain the cellular integrity. Moreover, the level of expression of these proteins could also be critical to produce the actin cytoskeletal reorganisations necessary to initiate HIV-induced membrane fusion events, viral assembly and budding [11, 12]. Inhibition of these mechanisms by endogenous Tat could be important to avoid the re-infection of current HIV-infected cells.

The identification and characterisation of the mechanism of action of these proteins could improve our knowledge of the HIV-1 life cycle, virus–host interactions and HIV-1 pathogenesis.

2 Materials and methods

2.1 Cells

A Jurkat cell line was cultured in RPMI 1640 medium (Biowhitaker, Walkersville, MD, USA) with 10% foetal calf serum (FCS) (PAN Biotech, Aidenbach, Germany), 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin, at 37°C. A Jurkat Tet-Off cell line was obtained from BD Biosciences Clontech (Mountain View, CA, USA) and cultured according to the manufacturer's instructions. Jurkat Tet-Off cells stably transfected with HIV-1 Tat protein were maintained in 300 μg/mL geneticin (Sigma-Aldrich, St. Louis, MO, USA) and 300 μg/mL hygromycin B (BD Biosciences Clontech). Tat expression was controlled by adding 1 μg/mL doxycycline (BD Biosciences Clontech) in the culture medium.

2.2 Vectors

Vector with viral *tat* gene from HIV-1 under the control of the cytomegalovirus promoter (CMV-Tat) was previously described [13]. Full-length Tat cDNA (aa 1–101) was obtained from CMV-Tat and cloned in pTRE2hyg vector (BD Tet-Off gene expression System, BD Biosciences) using *Bam*HI/*Nhe*I cloning sites. pTRE2hyg vector was used as negative control. The luciferase reporter gene under the control of the U3+R regions of the HIV-long terminal repeat (LTR) (LAI strain) was previously reported [14] (LTR-LUC). LTR-enhanced green fluorescent protein (EGFP) vector was generated cloning the full-length EFGP cDNA obtained from the pEGFP plasmid (BD Biosciences) in the LTR-LUC plasmid. The vector pNL 4.3 contains the complete HIV genome and induces an infectious progeny after transfection in several cell lines (National Institute of Health AIDS Research and Reference Reagent Program no. 3418). Plasmids were purified using Qiagen Plasmid Maxi Kit (Qiagen, CA, USA), following the manufacturer's instructions.

2.3 Antibodies

Mouse mAb to HIV-1 Tat regulatory protein (aa 1–16) was obtained from Advanced Biotechnologies (ABI, Columbia, MD, USA). Secondary antibody conjugated to Alexa 488 was purchased from Molecular Probes (Eugene, OR, USA).

2.4 Transfection assays

Stable transfection of Jurkat cells was performed by electroporation with an Easyjet Plus Electroporator (Equibio, Middlesex, UK). In brief, $3–5 \times 10^6$ cells were re-suspended in 250 μL of RPMI without supplements and then mixed with 1 μg of plasmid DNA per 10^6 cells in a 2-mm electroporation cuvette (Equibio). Cells were transfected by two pulses at 280 V, 150 μF, and 330 Ω. After transfection, cells were incubated in supplemental RPMI at 37°C for 24 h and then geneticin and hygromycin B were added. For transient transfections, $3–5 \times 10^6$ cells were re-suspended in 350 μL of RPMI without supplements and mixed with 1 μg of plasmid DNA per 10^6 cells in a 4-mm electroporation cuvette (Equibio). Cells were transfected at 280 V and 1500 μF in all cases. After transfection, cells were incubated in supplemental RPMI at 37°C for 24 h.

2.5 HIV-1 infection

MT-2 cells were infected with 50 ng of HIV-gag p24/10^6 cells. After 4 h of adsorption, cells were extensively washed and cultured for 7 days in RPMI 1640 medium (Biowhitaker) with 10% FCS (PAN Biotech), 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin, at 37°C.

2.6 Confocal microscopy and flow cytometry

For immunofluorescence assays, cells were immobilised in PolyPrep slides (Sigma-Aldrich) for 15 min and were fixed with 2% paraformaldehyde-0.025% glutaraldehyde in PBS for 10 min at room temperature. After washing twice with 0.1% glycine/PBS, cells were permeabilised with 0.1% Triton X-100/PBS for 10 min and then treated with 1 mg/mL $NaBH_4$ for 10 min. Incubation for 1 h at room temperature with each primary and secondary antibody and subsequent washes were performed with PBS/2% BSA/0.05% saponin buffer. Coverslips were immobilised with 70% glycerol/PBS. Images were obtained with a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA).

Cells transiently transfected with vector LTR-EGFP were analysed by gating live (propidium iodide-negative) cells and by size and complexity [15]. Cells were analysed on a FACScalibur Flow Cytometer (BD Biosciences), using CellQuest software (BD Biosciences).

2.7 Determination of nuclear DNA loss and cell cycle analysis

The percentage of cells undergoing chromatinolysis (subdiploid cells) was determined by ethanol fixation (70%, for 24 h at 4°C). The cells were then washed twice with PBS containing 4% glucose and subjected to RNA digestion (RNAse-A, 50 U/mL) and PI (20 μg/mL) staining in PBS for 1 h at room temperature, and analysed by cytofluorometry. With this method, low-molecular-weight DNA leaks from the ethanol-fixed apoptotic cells and the subsequent staining allows determination of the percentage of subdiploid cells (sub-G_0/G_1 fraction).

2.8 Detection of DNA strand breaks by the TUNEL method

Cells (1×10^6) were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, washed twice in PBS and permeabilised in 0.1% sodium citrate containing 0.1% Triton X-100 for 20 min. Fixed cells were washed three times in PBS and re-suspended in a final volume of 50 μL TUNEL buffer (0.3 nmol FITC-dUTP, 3 nmol dATP, 50 nmol $CoCl_2$, 5 U TdT, 200 mM potassium cacodylate, 250 μg/mL BSA and 25 mM Tris-HCl pH 6.6). The cells were incubated for 1 h at 37°C and then washed twice in PBS and analysed by flow cytometry.

2.9 Western blot assays

Nuclear protein extracts were obtained as described previously [16] and 50 μg of nuclear proteins was fractionated by SDS-PAGE and transferred onto Hybond-ECL NC paper (GE Healthcare, Uppsala, Sweden). After blocking and incubation with primary and secondary antibodies, proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

2.10 mRNA detection

Total RNA from MT-2 and Jurkat Tet-Off infected with NL 4.3, Jurkat-Tat, and negative controls was isolated with a RNeasy® Mini kit Ultraspec™ (Qiagen) following the manufacturer's recommendations. To that end, 2 μg total RNA from either of them was reverse transcribed in a total volume of 40 μL of the amplification buffer (50 mM Tris-HCl, pH 8,3, KCl, 10 mM $MgCl_2$, 0.5 spermidine and 10 mM DTT, 0.5 μL of each deoxynucleotide, 3.3 μM random hexamers) including RNAsin and ImProm-II™ Reverse transcription (Promega, Madison, WI, USA) at 1 U/μL. The mixture was incubated at 42°C for 60 min, followed by a 10-min incubation at 90°C, and the final volume was taken to 40 μL with water. For each sample an identical amount of RNA was incubated with (RT+) or without (RT−) reverse transcriptase. A CMV-Tat plasmid was used as positive control. Amplification of the *tat* gene was conduced on 8 μL of each cDNA synthesis reaction in 25 μL of solution containing 50 μM of each deoxynucleotide, 0.1 μM of each oligonucleotide primer and 2 U of Taq DNA polymerase (Promega). Amplification conditions were as follows: 95°C for 3 min, 35 cycles of 95°C for 30 s; 55°C for 30 s; 72°C for 1 min; and a 10-min extension step at 72°C. Primers used were: sense 5'GCAAGAA ATGGAGCCAGTAG3', antisense 5'GTACTA-CTTACTGCTTTGATAGAG3', amplifying a 215-pb product. PCR products were resolved on a 2% agarose gel.

2.11 2-D DIGE

2.11.1 Sample preparation

Sample extracts were prepared from cell suspensions washed with PBS. Pelleted cells were broken by mechanical disruption with the Sample Grinding Kit (GE Healthcare) with lysis buffer (30 mM Tris-HCl pH 8.5 (4°C), 6 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT) for 30–60 min and the supernatant was clarified by ultracentrifugation. Proteins were purified and concentrated by precipitation with the 2-D Clean-Up Kit (GE Healthcare), and re-suspended for 1–2 h in lysis buffer without DTT. When necessary the pH was adjusted to 8.5 at cool temperature. Protein concentration was measured with the RC/DC Protein Assay Kit (Bio-Rad). Samples were kept frozen until use.

2.11.2 Protein labelling with DIGE fluorophores

Protein samples were prepared and labelled according to the manufacturer's protocol. Briefly, 50 μg of each protein extract was labelled separately at 0°C in the dark for 30 min with 400 pmoles of the *N*-hydroxysuccinimide esters of cyanine dyes (Cy3 and Cy5 CyDyes; GE Healthcare) dissolved in 99.8% DMF (Sigma). The internal standard, the equimolecular mixture of all the protein extracts, was labelled with the Cy2 CyDye. The labelling reaction was quenched by the addition of 1 μL of a 10-mM L-lysine solution (Sigma) and left

on ice for 10 min. After labelling and quenching, the Cy2, Cy3 and Cy5 appropriate samples were mixed, and a volume of 2× rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS) containing 50 mM DTT and 1% IPG buffer was added.

2.11.3 2-DE with IPG strips

2-DE was performed as described elsewhere [17, 18], using precast IPG strips (pH 3–11 NL, non-linear, 24 cm length; GE Healthcare) in the first dimension (IEF). Samples were applied by cup-loading onto IPG strips previously rehydrated with 450 µL rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.75% IPG buffer pH 3–11 NL, 100 mM DeStreak (GE Healthcare) and minute amounts of bromophenol blue). Typically, 100–150 µg protein was loaded on each IPG strip and IEF was carried out with the IPGPhor II (GE Healthcare) following a voltage stepwise increase of 300 V for 3 h, gradient increase from 300 to 1000 V for 4 h, gradient increase from 1000 to 8000 V for 2 h, and constant 8000 V until the steady-state. After IEF separation, the strips were equilibrated twice for 15 min with 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS and a trace of bromophenol blue. The first equilibration solution contained 2% DTT, and 4% iodoacetamide was added in the second equilibration step instead of DTT. Second-dimension SDS-PAGE was performed using 1-mm-thick, 26 × 22-cm, 12.5% homogeneous polyacrylamide gels, and electrophoresis was carried out at a constant current (45 min at 2 mA/gel and about 4 h at 20 mA/gel) and temperature (25°C).

2.11.4 Image acquisition and analysis

After electrophoresis, gels were scanned using the Typhoon 9400 Imager (GE Healthcare) according to the manufacturer's protocol. Scans were acquired at 100 µm resolution. After cropping, images were subjected to automated Difference In-gel Analysis (DIA) and Biological Variation Analysis (BVA) using DeCyder software version 5.01 (GE Healthcare).

2.12 In-gel digestion of proteins

Protein spots from silver-stained gels were excised manually and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany). The digestion protocol used was that of Schevchenko *et al.* [19], with minor variations: gel plugs were submitted to reduction with 10 mM DTT (GE Healthcare) in 50 mM ammonium bicarbonate (99.5% purity; Sigma-Aldrich) and alkylation with 55 mM iodoacetamide (Sigma-Aldrich) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and ACN (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega) at a final concentration of 13 ng/µL in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37°C for 6 h. Finally, 0.5% TFA (99.5% purity; Sigma-Aldrich) was added for peptide extraction.

2.13 MALDI-MS(/MS) and database searching

An aliquot of the above digestion solution was mixed with an aliquot of CHCA (Bruker-Daltonics) in 33% aqueous ACN and 0.1% TFA. This mixture was deposited onto a 600-µm AnchorChip MALDI probe (Bruker-Daltonics) and allowed to dry at room temperature. MALDI-MS(/MS) data were obtained using an Ultraflex TOF/TOF mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device [20]. Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100 to 1500 individual spectra were averaged. For fragment ion analysis in the TOF/TOF mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analysed after passing the ion reflector. Measurements were in part performed using post-LIFT metastable suppression, which allowed removal of precursor and metastable ion signals produced after extraction out of the second ion source. Peptide mass mapping data were analysed in detail using flexAnalysis software (Bruker-Daltonics). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800–3200 m/z region. MALDI-MS and MS/MS data were combined through the MS BioTools program (Bruker-Daltonics) to search the NCBInr database using MASCOT software (Matrix Science, London, UK) [21].

3 Results

3.1 Analysis of Tat subcellular distribution in stably transfected Jurkat Tet-Off cells

HIV-1 Tat protein presents a nuclear localisation sequence that is able to confer nuclear entry and binding to nuclear components, therefore promoting accumulation of HIV-Tat in the nucleus [22, 23]. Jurkat Tet-Off cells stably transfected with pTRE2hyg-Tat vector were analysed by intracellular staining using a mAb against Tat (aa 1–16). Confocal microscopy observations revealed basically a nuclear localisation for this protein (Fig. 1). This result has been confirmed by Western blot analysis of the nuclear fraction using anti-Tat antibodies (Fig. 2A).

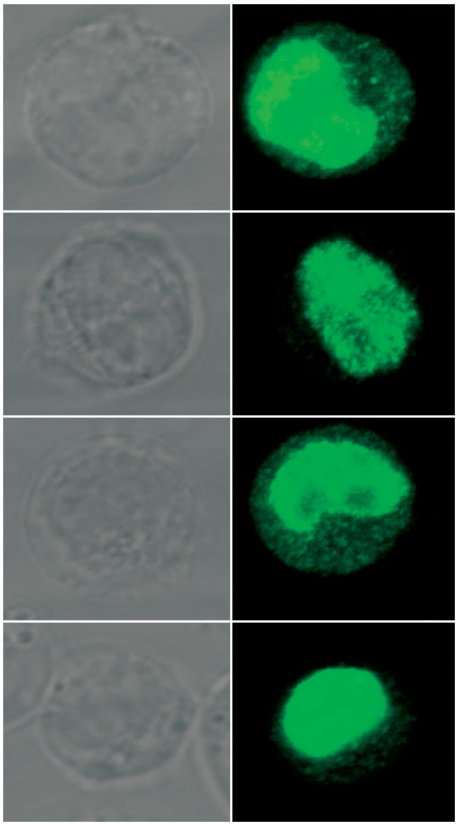

Figure 1. Subcellular localisation of Tat in stably transfected Jurkat cells. Cells were fixed, permeabilised and stained with a mAb against Tat (aa 1–16). A secondary antibody conjugated with Alexa 488 (Molecular Probes) was used. Images were obtained by confocal microscopy as described in Section 2.

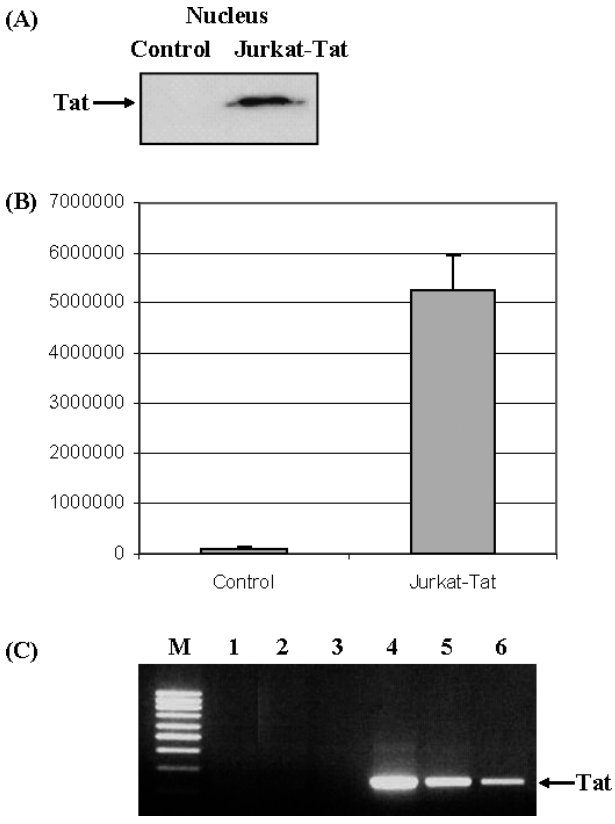

Figure 2. (A) Analysis of the presence of Tat in the nucleus of Jurkat-Tat cells. Fifty micrograms of nuclear extracts was analysed by Western blot using a mAb against Tat (aa 1–16). (B) Transcriptional activity of nuclear Tat expressed in Jurkat-Tat cells compared to Jurkat Tet-Off cells as negative control. Cells were transfected by electroporation with 1 μg of LTR-LUC vector per million by electroporation. Analysis of Tat transcriptional activity was performed 18 h after transfection by measuring luciferase activity. Data normalisation was performed by protein concentration measurement. (C) mRNA Tat levels as determined by RT-PCR in water control (lane 1), non-infected cells (lane 2), control Tet-Off cells (lane 3), HIV-infected MT-2 cells (lane 4), CMV-Tat plasmid as positive control (lane 5), and Jurkat-Tat cells (lane 6). Arrow indicates position of 251-pb Tat amplicons.

3.2 Expression and functionality of Tat protein

Once the presence of Tat in the nucleus of Jurkat-Tat cells was determined, the capacity of binding and transactivating the HIV LTR promoter was examined. The transcriptional capacity was analysed by transient transfection of the Jurkat-Tat cells with vector LTR-LUC. In the absence of virally encoded Tat there is little or no gene expression directed by the proviral LTR [24, 25]; in contrast, the presence of Tat triggers gene expression. Figure 2B shows the luciferase activity that was obtained in Jurkat-Tat cells in comparison with Jurkat Tet-Off as control. Therefore, a correlation between the percentage of Tat-expressing cells and its activity in different clones or in the whole population was found. These data demonstrate that Tat is functional in this system.

We also analysed whether Tat expression was physiological in our model in comparison to Tat levels observed in HIV-infected cells. To this aim, Tat transcripts were analysed by RT-PCR. As shown in Fig. 2C, mRNA levels were similar in cells stably transfected with a Tat-expressing vector and HIV-infected cells.

3.3 Analysis of expression percentage of Tat protein

To determine the percentage of Jurkat cells that expressed Tat within the whole population of stable transfectants, the vector LTR-EGFP was transiently transfected in these cells and EGFP expression was analysed by flow cytometry. As shown in Fig. 3, Jurkat-Tat cells were a mixed population in which 35% of cells presented a high expression of Tat.

3.4 Analysis of cell cycle phases and apoptosis

Although HIV-1 Tat has been related to increased apoptosis in infected cells [26, 27], this is unclear in cells expressing Tat exclusively. Moreover, the apoptosis pattern has been studied

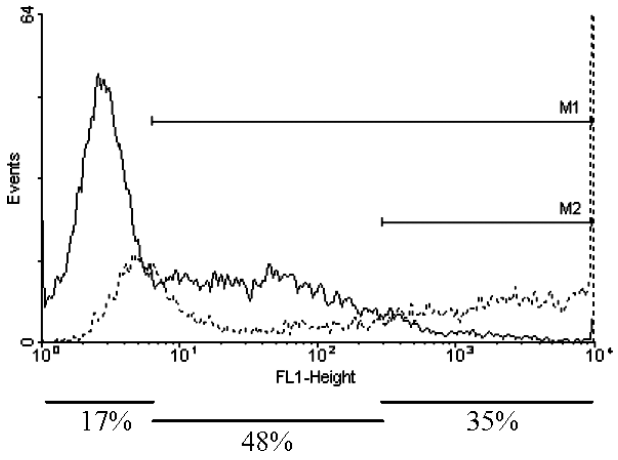

Figure 3. Quantification of population of Jurkat-Tat cells expressing functional Tat by flow cytometry. Cells were transfected by electroporation with 1 μg of LTR-EGFP vector per million by electroporation. Analysis of fluorescence in channel FL1 was performed 18 h after transfection by flow cytometry. Continuous line shows the fluorescence pattern for Jurkat Tet-Off cells used as control. Discontinuous line shows the fluorescence pattern for Jurkat-Tat cells. Percentages indicated below the histogram correspond to three different populations.

in a wide variety of Tat-expressing cell lines and no increase of apoptosis was observed [28]. Because Jurkat-Tat cells expressed high levels of functional Tat, we examined how the accumulation of Tat protein in the nucleus influenced these cells. The analysis of the cell cycle of Jurkat-Tat cells related to Jurkat Tet-Off cells showed no variation in sub-G_0 and G_1/S phases, although a slight decrease in the G_2/M phase was observed in Jurkat-Tat cells (Fig. 4A). The anti-apoptotic effect of Tat in the Jurkat cells was analysed by flow cytometry using two different methods: the measurement of apoptotic-mediated DNA degradation by propidium iodide staining (Fig. 4A) and enzymatic labelling of DNA strand breaks with FITC-dUTP by TUNEL assay (Fig. 4B). The apoptosis was induced by addition to the culture medium of 0.1 and 1 μg/mL of tunicamycin, an ER stress agent. Data showed a decrease of apoptosis in Jurkat-Tat as compared to the control by both methods.

3.5 Analysis of cell proteome by DIGE and MS

The proteomic analyses allowed the identification of proteins with altered expression levels in Jurkat-Tat cells using Jurkat Tet-Off cells as negative control. Proteins with significant varying expression levels were identified by MS analysis. To achieve an accurate determination of these varying proteins, three DIGE experiments were performed using three different batches of Jurkat-Tat cells expressing high levels of functional Tat. Figure 5 shows a representative DIGE analysis of Jurkat Tet-Off cell proteins used as control (Fig. 5A) as well as the corresponding Jurkat-Tat cells (Fig. 5B). Table 1 lists the identification results for those proteins showing in all samples replicates with a distinct expression pattern. Several other proteins were identified in the different DIGE gels but not with enough reproducibility in all experiments (data not shown). Therefore, these proteins were not included in this final report. All proteins described in Table 1 were successfully identified by MS analysis and were found to have decreased expression levels in the Jurkat-Tat cells compared to Jurkat Tet-Off cells: all proteins were cytoskeletal (β-tubulin, β-actin, cytoplasmic actin, and annexin II) or involved in cytoskeletal reorganisation (cofilin 1, gelsolin isoform b, and Rac/Rho-GDI complex).

4 Discussion

HIV-1 infection is associated with depletion of CD_4^+ T cells. However, these cells also constitute one of the major viral reservoirs and there are subsets of long-lived producers of competent virus in a small number of CD_4^+ T cells that survives to apoptosis. The mechanisms involved in HIV-1 infection persistence remain unclear.

HIV-1 Tat protein is a potent transactivator not only essential for viral replication, but also able to transactivate some cellular genes [10]. In fact, in addition to the direct killing of the infected cells, HIV infection also leads to increased apoptosis of predominantly uninfected bystander cells. Viral proteins such as Env, Vpr and Tat are described as mediators of this effect. Tat acts not only at an intracellular level in HIV-infected cells but also as a soluble protein. In fact, Tat is secreted by the infected cells and taken up by uninfected cells where it translocates to the nucleus in an active form [7] and transactivates different cellular genes [8, 9]. Soluble Tat has been described as highly toxic, since it causes dramatic actin cytoskeletal rearrangements such as membrane ruffling, peripheral retraction and cytoskeletal disassembly that conclude in cell death [2]. Therefore, it could be considered as a pro-apoptotic factor [29–32]. However, there is contradictory evidence that Tat could also present anti-apoptotic properties [33, 34].

In order to study the proteomic changes that can be induced by Tat expression in CD_4^+ T cells, a Jurkat cell line that constitutively expresses Tat has been developed. Because Tat has often been described as highly toxic in cells, we developed Jurkat-Tat cells using a repressible Tet-Off expression system that allows the expression of Tat to be turned off with the aim of assuring the continuation of its expression. No study has been previously performed using a stable Tet-Off Tat expressing system that allows the study of the effect of Tat on T cells minimising its toxicity. Analysis of these cells by confocal microscopy showed that Tat accumulates predominantly in the nucleus (Figs. 1 and 2A) as previously described [22, 23]. Functional experiments also proved that a majority of the population was expressing high levels of Tat (see Fig. 3) and that this protein was able to transactivate the HIV LTR (Figs. 2B and 3). In our cell model, physiological

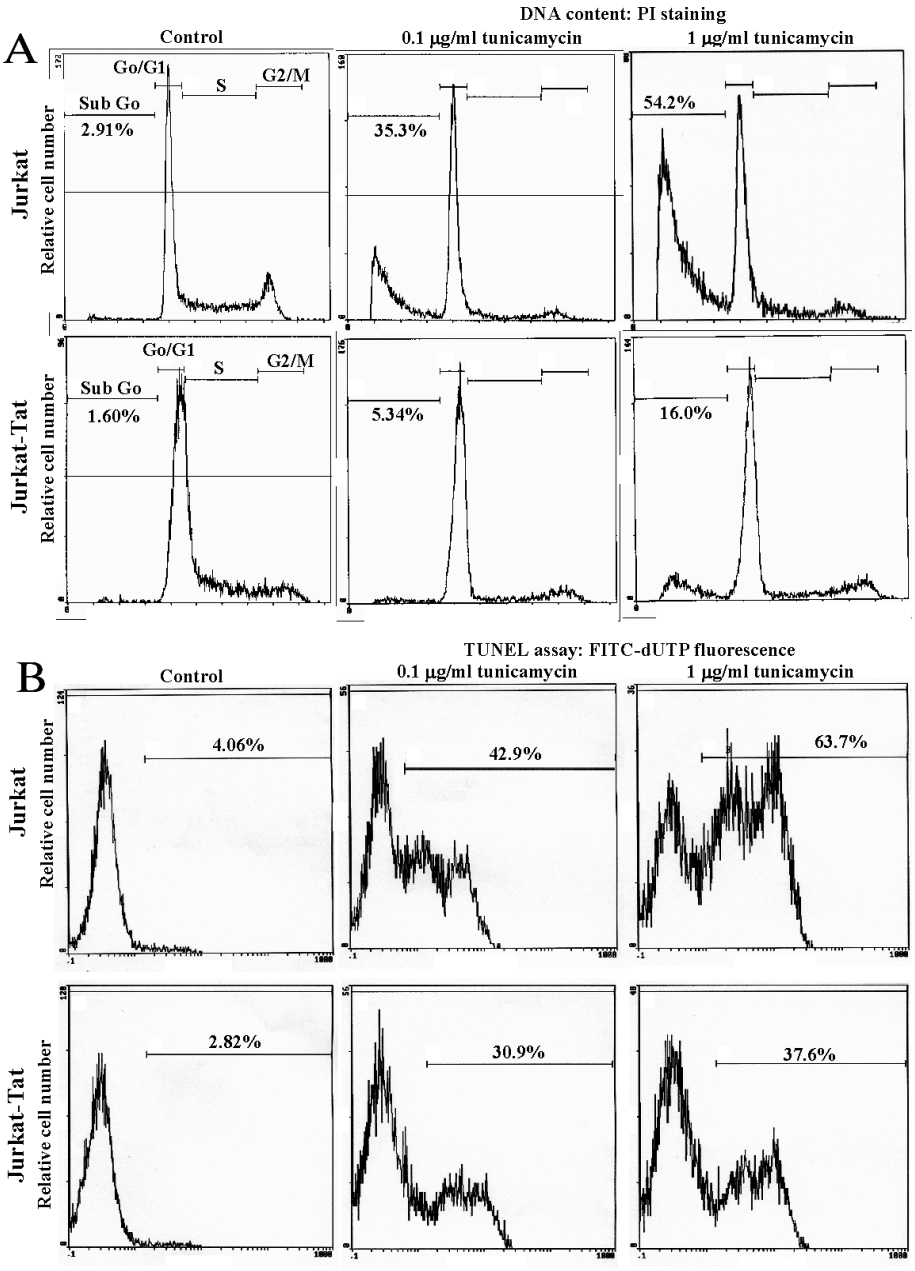

Figure 4. Quantification of apoptotic effect caused by 0.1 and 1 μg/mL tunicamycin in Jurkat-Tat cells and Jurkat Tet-Off cells used as negative control by propidium iodide staining (A) and TUNEL assay (B).

levels of Tat were produced because mRNA expression was similar in cells stably transfected with a Tat-expressing vector and HIV-infected cells (Fig. 1C). Intracellular Tat expressed in human T cells was scarcely toxic to these cells (see Fig. 4A). In fact, a quite moderate twofold decrease of the G_2/M phase was observed in Jurkat-Tat cells compared with the Jurkat Tet-Off used as control. No other significant changes in the cell cycle pattern were observed.

The role of Tat in HIV-induced apoptosis remains controversial. Indeed, both pro-apoptotic and anti-apoptotic activities of Tat protein have been reported. Intracellular Tat expressed in stable cell lines was able to protect these cells from cellular injury [35], whereas soluble Tat acts as a toxin at distant sites [36]. Our results support previous studies showing that there is no significant increase of apoptosis in Tat-expressing lymphocytes [28]. Furthermore, experiments performed to quantify the possible apoptotic effect of Tat in the transfected Jurkat cells showed a protective effect against apoptosis induced by a stress agent such as tunicamycin (Fig. 4B).

Therefore, we conclude that although the infection of HIV-1 has profound consequences in the cellular status, such as the phase of the cell cycle, the stage of cell differentiation and cell death, those changes could not be produced exclusively by Tat expression, at least in lymphocytes.

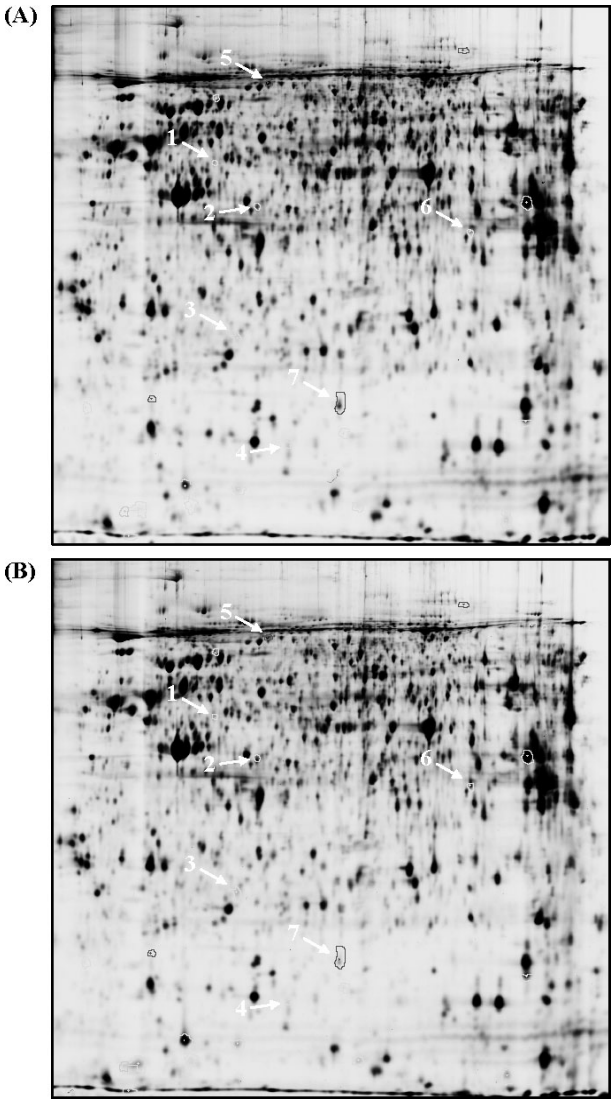

Figure 5. Representative DIGE shows proteome analyses of Jurkat Tet-Off cell (A) and Jurkat-Tat cells (B). Main differences of protein expression are circled and arrows indicate spots considered for the present study. Spots are numbered according to Table 1. The first dimension was run on IPG strips pH 3–11 NL and the second dimension on 12% polyacrylamide gels as described in Section 2.

Given that HIV-1 has been demonstrated to either up- or down-regulate gene expression [36–39], a proteomic analysis was performed by DIGE and MS to determine whether the single expression of Tat could induce changes in the proteome of these Jurkat-Tat cells. Seven major different proteins involved mainly in cytoskeletal activity were found to be down-expressed in these Jurkat-Tat cells (Table 1). Three different batches of constitutive Tat-expressing Jurkat cells were employed to confirm the presence of these protein species with significantly varying expression levels. Altered expression levels were found in basic cytoskeletal proteins such as actin, β-tubulin, annexin, cofilin and gelsolin, as well as in the Rac/Rho-GDI complex; the latter was also involved in signal transduction and cellular communication. All these proteins were found to have decreased expression in Jurkat-Tat cells compared to control.

The cell cytoskeleton in eukaryotic cells consists of three interconnected filament systems: microfilaments consisting of actin, microtubules made from α/β-tubulin subunits, and intermediate filaments comprising fibrous proteins. HIV uses the cytoskeletal system for infection and replication. The cytoskeleton is important for virion assembly, and reverse transcription is dependent on an intact actin network [40, 41]. The interaction between actin and viral particle formation appears to be mediated by Gag [42], and the decrease in actin expression in Jurkat-Tat cells could affect proper virion assembly. On the other hand, α/β-tubulin is known to form heterodimers and function as the building blocks of microtubules, along with actin. Tat binds α/β-tubulin dimer and polymerised microtubules in the cytoplasm of the cell through its evolutionary conserved core region, and this interaction alters microtubule dynamics preventing microtubule depolymerisation [43]. Interaction between Tat and tubulin also delays the polymerisation of tubulin and induces a premature stop to microtubule-dependent cytoplasmic streaming [44].

On the other hand, while actin is the major component of the cytoskeleton, other cytoskeletal proteins are associated with actin. These proteins bind and regulate the polymerisation of actin, while other proteins cross-link actin filaments to form fibres and to link the cytoskeleton to the plasma membrane [45]. Within these proteins there is cofilin, an actin-binding and -depolymerising protein [46], as well as gelsolin. The gelsolin superfamily proteins control actin organisation by dissolving filaments, capping filament ends and nucleating actin assembly [47]. These proteins present a role in actin filament remodelling and also have specific roles in several cellular processes such as cell motility and control of apoptosis. In this work, decreased levels of actin, tubulin, cofilin and gelsolin in Jurkat-Tat cells compared to control are reported. Binding of Tat to these proteins appears to be crucial to activate a mitochondrial-dependent apoptotic pathway [43]. Thus, decreased levels of β-tubulin in T cells may stabilise the cell and explain the absence of apoptosis observed in the Jurkat-Tat cells despite the high expression of functional Tat. In fact, it could explain the survival of certain subsets of HIV-infected CD_4^+ T cells leading to long-term production of HIV.

Annexin II has been described as a cellular cofactor supporting macrophage HIV-1 infection [48]. Annexin II mediates interactions between cholesterol-rich membrane domains and the actin cytoskeleton, which may navigate HIV-1 through the labyrinth of the cell membrane [49]. Annexin II interacts with the actin cytoskeleton and it might not only facilitate HIV-1 internalisation, but also the trafficking of the virus within the cell [50]. Therefore, annexin II has been described as a potential cofactor that might be usurped by HIV-1 to facilitate the infectious process. In fact,

Table 1. Summary of proteins identified by MALDI-MS(/MS)

Spot no.	MASCOT score	Accession code	Name	M_r	p*I*	Peptide sequences[a)]
1	190	gi|18088719	β-Tubulin	50	4.5	YLTVAAVFR IREEYPDR FPGQLNADLR LAVNMVPFPR ISEQFTAMFR FPGQLNADLRK ISVYYNEATGGK AILVDLEPGTMDSVR ALTVPELTQQVFDAK EIVHIQAGQCGNQIGAK GHYTEGAELVDSVLDVVR
2	136	gi|49868	β-Actin	39	5.8	AVFPSIVGR GYSFTTTAER PRAVFPSIVGR IWHHTFYNELR QEYDESGPSIVHR SYELPDGQVITIGNER VAPEEHPVLLTEAPLNPK
3	195	gi|52854308	Cytoplasmic actin	26	5.2	IIAPPERK GYSFTTTAER QEYDESGPSIVHR SYELPDGQVITIGNER
4	95	gi|9955206	Rac/Rho-GDI complex	21	6.2	YVQHTYR ATFMVGSYGPRPEEYEFLTPVEEAPK
5	242	gi|38044288	Gelsolin isoform b	81	5.5	TGAQELLR EPGLQIWR EGGQTAPASTR YIETDPANR VVEHPEFLK AGKEPGLQIWR HVVPNEVVVQR AGALNSNDAFVLK YIETDPANRDR SEDCFILDHGKDGK EVQGFESATFLGYFK QTQVSVLPEGGETPLFK TPSAAYLWVGTGASEAEK AQPVQVAEGSEPDGFWEALGGK DPDQTDGLGLSYLSSHIANVER VPFDAATLHTSTAMAAQHGMDDDGTGQK
6	93	gi|9247201	Annexin II	39	7.5	QDIAFAYQR TNQELQEINR QDIAFAYQRR SNAQRQDIAFAYQR AEDGSVIDYELIDQDAR
7	148	gi|62751777	Cofilin 1 (non-muscle)	19	8.2	AVLFCLSEDKK YALYDATYETK HELQANCYEEVK HELQANCYEEVKDR

a) Peptides subjected to MS/MS analysis are underlined

annexin II is involved in microbial entrance and pathogenesis of other viral pathogens such as cytomegalovirus and respiratory syncytial virus [51, 52].

Besides, the Rac/Rho-GDI complex expression was also found to be down-regulated in Jurkat-Tat cells. The GTP-binding protein Rac is essential to the activation of the

NADPH oxidase complex, involved in pathogen killing during phagocytosis. In resting T cells, Rac exists as a heterodimeric complex with Rho GDP dissociation inhibitor (Rho-GDI) [53]. During chemotaxis the actin cytoskeleton controls the polymerisation, orientation and forward motility of cells, primarily by responding to the regulatory Rho-family GTPases Rac and Rho [54]. In HIV-infected cells, HIV Env-coreceptor interactions activate Rac-1 GTPase and stimulate the actin filament network reorganisations that are requisite components of the cell fusion process [55]. Our findings showed that both annexin II and the Rac/Rho-GDI complex are down-expressed in Jurkat-Tat cells and it could be explained as a barrier to avoid re-infection of an HIV-infected CD_4^+ T cell. Therefore, the regulation of the expression of these proteins could be critical to produce the actin cytoskeletal reorganisations in HIV-induced cell–cell fusion. The membrane fusion events initiate the HIV infections and promote the characteristic cytopathic syncytium formation in infected cells.

In conclusion, some viral proteins such as Tat interact with cytoskeletal proteins causing profound cytoskeletal reorganisations in the host cell. Those changes can induce apoptosis. However, if a protection against apoptosis is proposed for Tat, the down-expression of cytoskeletal proteins and other proteins involved in actin depolymerisation could avoid or control these cytoskeletal reorganisation and therefore maintain the cellular integrity. This mechanism would permit that HIV-infected cells act as long-term reservoirs and continuous producers of HIV virions. Moreover, the down-expression of these proteins would be important to avoid the re-infection of present HIV-infected cells. In fact, there have been descriptions of some protection against infection in cells that are currently infected.

Accordingly, the current proteomic study is an attempt to define those cellular genes that Tat could critically regulate in a lymphocytic environment. The proteins that were down-regulated in Jurkat-Tat cells are mainly involved in cytoskeleton properties and may be critical in understanding how HIV uses CD_4^+ T cells as primary hosts, without destruction during a long period. Further studies will be needed to investigate the relationship of the proteins identified and the kinetics of HIV replication in human CD_4^+ T cells.

In conclusion, human CD_4^+ T lymphocytes are natural targets of HIV-1 and they constitute one of the major long-term reservoirs for these viruses. In an attempt to determine how the viral Tat protein can be involved in the mechanism to develop these long-lived viral producers, we developed a Jurkat cell line that expresses intracellular Tat in a constitutive way. Proteomic analysis showed that in Jurkat-Tat cells there was a down-regulation of certain cytoskeletal proteins. This fact along with the protection from apoptosis observed in these cells may explain how Tat could contribute to cell survival and increased viral production.

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5 References

[1] Gil, J., Bermejo, M., Alcami, J., in: Alonso, C. (Ed.), *Viruses and Apoptosis*, Springer Verlag, Berlin 2004, pp. 117–149.

[2] Wu, R. F., Gu, Y., Xu, Y. C., Mitola, S. *et al.*, *J. Virol.* 2004, *78*, 779–789.

[3] Finkel, T. H., Tudor-Williams, G., Banda, N. K., Cotton, M. F. *et al.*, *Nat. Med.* 1995, *1*, 129–134.

[4] Yin, J., Chen, M. F., Finkel, T. H., *AIDS* 2004, *18*, 587–596.

[5] Luciw, P. in: Fields, B. N., Knipe, D. M., and Howley, P. M. (Eds.), *Human Immunodeficiency Viruses and Their Replication*, Lippincott-Raven, Philadelphia 1996, pp. 1891–1952.

[6] Liang, W.S., Maddukuri, A., Teslovich, T. M., de la Fuente, C. *et al.*, *Retrovirology* 2005, *2*, 20–43.

[7] Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V. *et al.*, *J. Virol.* 1993, *67*, 277–287.

[8] Scala, G., Ruocco, M.R., Ambrosino, C., Mallardo, M. *et al.*, *J. Exp. Med.* 1994, *179*, 961–971.

[9] Westendorp, M. O., Li-Weber, M., Frank, R. W., Krammer, P. H., *J. Virol.* 1994, *68*, 4177–4185.

[10] Watson, K., Edwards, R. J., *Biochem. Pharmacol.* 1999, *58*, 1521–1528.

[11] Wang, E., Wolf, B. A., Lamb, R. A., Choppin, P. W. *et al.*, in: Goldman, R., Pollard, R., Rosenbaums, T. J. (Eds.), *Cell Motility*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1976, pp. 589–599.

[12] Damsky, C. H., Sheffield, J. B., Tuszynski, G. P., Warren, L., *J. Cell Biol.* 1977, *75*, 593–605.

[13] Madore, S. J., Cullen, B. R., *J. Virol.* 1993, *67*, 3703–3711.

[14] Bachelerie, F., Alcamí, J., Arenzana-Seisdedos, F., Virelizier, J. L., *Nature* 1991, *350*, 709–712.

[15] Ormerod, M. G., *Flow Cytometry: A Practical Approach*, Oxford University Press, New York 1992.

[16] Lain de Lera, T., Folgueira, L., Martín, A. G., Dargemont, C. *et al.*, *Oncogene* 1999, *18*, 1581–1588.

[17] Görg, A., Weiss, W., Dunn, M. J., *Proteomics* 2005, *4*, 3665–3685.

[18] Rabilloud, T., *Electrophoresis* 1998, *19*, 758–760.

[19] Schevchenko, A., Wilm, M., Vorm, O., Mann, M., *Anal. Chem.* 1996, *68*, 850–858.

[20] Suckau, D., Resemann, A., Schuerenberg, M., Hufnagel, P. *et al.*, *Anal. Bioanal. Chem.* 2003, *376*, 952–965.

[21] Perkins, D. N., Pappin, D. J., Creasy, D. M., Cottrell, J. S., *Electrophoresis* 1999, *20*, 3551–3567.

[22] Hauber, J., Malim, M., Cullen, B., *J. Virol.* 1989, *63*, 601–608.

[23] Efthymiadis, A., Briggs, L. J., Jans, D.A., *J. Biol. Chem.* 1998, *273*, 1623–1628.

[24] Kao, S. Y., Calman, A. F., Luciw, P. A., Peterlin, B. M., *Nature* 1987, *330*, 489–493.

[25] Feinberg, M. B., Baltimore, D., Frankel, A. D., *Proc. Natl. Acad. Sci. USA* 1991, *88*, 4045–4049.

[26] Seve, M., Favier, A., Osman, M., Hernandez, D. *et al.*, *Arch. Biochem. Biophys.* 1999, *361*, 165–172.

[27] Wang, P., Barks, J. D., Silverstein, F. S., *Neuroscience* 1999, *88*, 585–597.

[28] Clark, E., Santiago, F., Deng, L., Chong, S. Y. *et al.*, *J. Virol.* 2000, *74*, 5040–5052.

[29] Li, C. J., Friedman, D. J., Wang, C., Metelev, V. *et al.*, *Science* 1995, *268*, 429–431.

[30] Westendorp, M. O., Frank, R., Ochsenbauer, C., Stricker, K. *et al.*, *Nature* 1995, *375*, 497–500.

[31] Haughey, N. J., Mattson, M. P., *J. Acquir. Immune Defic. Syndr.* 2002, *31*, S55–61.

[32] Kim, T. A., Avraham, H. K., Koh, Y. H., Jiang, S. *et al.*, *J. Immunol.* 2003, *170*, 2629–2637.

[33] McCloskey, T. W., Ott, M., Tribble, E., Khan, S. A. *et al.*, *J. Immunol.* 1997, *158*, 1014–1019.

[34] Gibellini, D., Re, M. C., Ponti, C., Maldini, C. *et al.*, *Cell Immunol.* 2001, *207*, 89–99.

[35] Chauhan, A., Turchan, J., Pocernich, C., Bruce-Keller, A. *et al.*, *J. Biol. Chem.* 2003, *278*, 13512–13519.

[36] Pocernich, C. B., Boyd-Kimball, D., Poon, H. F., Thongboonkerd, V. *et al.*, *Mol. Brain Res*, 2005, *133*, 307–316.

[37] Su, Z.Z., Kang, D.C., Chen, Y., Pekarskaya, O. *et al.*, *Oncogene* 2002, *21*, 3592–3602.

[38] Galey, D., Becker, K., Haughey, N., Kalehua, A. *et al.*, *J. Neurobiol.* 2003, *9*, 358–371.

[39] Su, Z. Z., Kang, D. C., Chen, Y., Pekarskaya, O. *et al.*, *J. Neurovirol.* 2003, *9*, 372–389.

[40] Soll, D. R., *Cell Motil. Cytoskelet.* 1997, *37*, 91–97.

[41] Bukrinskaya, A., Brichacek, B., Mann, A., Stevenson, M., *J. Exp. Med.* 1998, *188*, 2113–2125.

[42] Mouland, A. J., Mercier, J., Luo, M., Bernier, L. *et al.*, *J. Virol.* 2000, *74*, 5441–5451.

[43] Chen, D., Wang, M., Zhou, S., Zhou, Q., *EMBO J.* 2002, *21*, 6801–6810.

[44] Battaglia, P. A., Zito, S., Macchini, A., Gigliani, F., *Curr. Opin. Neurobiol.* 2001, *11*, 387–394.

[45] Pollard, T. D., Cooper, J. A., *Annu. Rev. Biochem.* 1986, *55*, 987–1035.

[46] Ogawa, K., Tashima, M., Yumoto, Y., Okuda, T. *et al.*, *Nucleic Acids Res.* 1990, *18*, 7169.

[47] Silacci, P., Mazzolai, L., Gauci, C., Stergiopulos, N. *et al.*, *Cell Mol. Life Sci.* 2004, *61*, 2614–2623.

[48] Ma, G., Greenwell-Wild, T., Lei, K., Wenwen, J. *et al.*, *J. Exp. Med.* 2004, *200*, 1337–1346.

[49] Mayran, N., Parton, R. G., Gruenberg, J., *EMBO J.* 2003, *22*, 3242–3253.

[50] Chan, D. C., Kim, P. S., *Cell* 1998, *93*, 681–684.

[51] Raynor, C. M., Wright, J. F., Waisman, D. M., Pryzdial, E. L., *Biochemistry* 1999, *38*, 5089–5095.

[52] Malhotra, R., Ward, M., Bright, H., Priest, R. *et al.*, *Microbes Infect.* 2003, *5*, 123–133.

[53] Di-Poi, N., Faure, J., Grizot, S., Molnar, G. *et al.*, *Biochemistry* 2001, *40*, 10014–10022.

[54] Eitzen, G., *Biochim. Biophys. Acta* 2003, *1641*, 175–181.

[55] Pontow, S. E., Vander Heyden, N., Wei, S., Ratner, L., *J. Virol.* 2004, *78*, 7138–7147.

