Virus Genes (2010) 40:341–346
DOI 10.1007/s11262-010-0458-7

Over-expression of the HIV-1 Rev promotes death of nondividing eukaryotic cells

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Received: 22 September 2009 / Accepted: 30 January 2010 / Published online: 12 February 2010

Abstract Expression of the human immunodeficiency virus type 1 (HIV-1) Rev protein is essential for completion of the viral life cycle. Rev mediates nuclear export of partially spliced and unspliced viral transcripts and therefore bears a nuclear localization signal (NLS) as well as a nuclear export signal (NES), which allow its nucleocytoplasmic shuttling. Attempts to express the wild-type Rev protein in eukaryotic human cultured cells have encountered difficulties and so far have failed. Here we show that accumulation of Rev, which occurs in nondividing Rev-expressing cells or when such cells reach confluency, results in death of these cells. Cell death was also promoted by addition of a cell permeable peptide bearing the Rev-NES sequence, but not by the Rev-NLS peptide. Our results probably indicate that binding of excess amounts of the Rev protein or the NES peptide to the exportin receptor CRM1 results in cells' death.

Keywords Rev · HIV-1 · Stable expression · Cell death · Nucleocytoplasmic shuttling

Introduction

The human immunodeficiency virus type 1 (HIV-1) Rev is a 13.5 kDa protein containing 116 residues [1]. It bears a nuclear localization signal (NLS) as well as a nuclear export signal (NES), which allow its shuttling between the cytoplasm and nuclei of HIV-1 infected cells. Rev is an oligomeric protein that forms multimers in vitro [2] as well as in vivo [3]. The nuclear import of Rev is due to the presence of the NLS (residues 35–50), which mediates its binding to the cellular receptor importin β [4–7]. The Rev NLS contains several arginine residues and therefore has been designated also as an argenine rich motif (ARM) [4]. An additional function of the ARM is to mediate the binding of Rev to a specific 240 nucleotides RNA sequence, termed the Rev responsive element (RRE). This interaction allows Rev-mediated nuclear export of partially spliced and unspliced viral RNA [4, 8]. The nuclear export ability of the Rev is due to the presence of the NES motif (residues 75–84), which is characterized by the presence of several leucine residues [4, 9] and is recognized by the cellular receptor CRM1/exportin 1 [10, 11].

To fully understand the biological functions of Rev, it is of importance to study its structure–function relationship. Such studies should be carried out by using a full-length purified wild-type (WT) Rev. However, attempts to express a functional soluble WT Rev in prokaryotic cells have encountered difficulties for a long time, and therefore, it was traditionally used as a Rev–GFP conjugant [12, 13]. Recently, two articles describing the expression and purification of untagged Rev protein have been published [14, 15]. Expressing full-length WT Rev in human eukaryotic cells also failed so far, and it was mostly obtained as a Rev–GFP fusion protein as in prokaryotic cells [16]. A stably expressed Rev was obtained only in the African green monkeys-derived COS cultured cells [17]. The Rev M10 mutant, lacking nuclear export activity [18], could be expressed also in human eukaryotic cells [18–20].

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Here we show that the failure to obtain a stably transfected human Rev-expressing cell line is due to the fact that in confluent or nondividing cells, accumulation of Rev leads to cell death. On the other hand, expressing Rev in dividing, nonconfluent, human cultured cells does not result in cell death. Attempts to explain the molecular mechanism, which promotes cell death of Rev-expressing human cells, are described.

Materials and methods

Cells

Monolayer adherent HeLa and HEK293T were grown in Dulbecco's Modified Eagle's Medium (DMEM). The T-lymphocyte cell lines H9 were grown in RPMI 1640 medium. Cells were provided by the NIH Reagent Program, Division of AIDS, NIAID, NIH, USA and were incubated at 37°C in a 5% CO_2 atmosphere. All media were supplemented with 10% (v/v) fetal calf serum, 0.3 g/l L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Biological Industries, Beit Haemek, Israel).

Rev expression vectors

The Rev and the Rev M10 genes were amplified by PCR using the forward primer 5′-AACTAGCTAGCATGG CAGGAAGAAGCGGAGACAG-3′ and the reverse primer 5′-ATATGTCGACTCACATTCTCTTTAGTTCCTGACT CGAG-3′. After amplification, the Rev genes were inserted into the pcDNA 3.1/Hygro(+) plasmid (Invitrogen) using the restriction enzymes *Nhe*I and *Xba*I.

Transfection of cultured cells with the Rev bearing vectors

Transfection of HEK293T and HeLa cell was preformed essentially as described before [21] using cells monolayer that reached about 40–50% confluency. Transfection of H9 lymphocytes was preformed as described before [22]. Selection was carried out for 4 weeks using 400 μg/ml Hygromycin B. Transfected monolayer cells were split before reaching 60% confluency.

Western blot analysis

Cells were harvested, washed three times with PBS, and lysed by the addition of PBS containing 1% (v/v) Triton X100. The lysate was subjected to an SDS PAGE, immunoblotted, and detected with a monoclonal anti-Rev antibody at 1:50 [23] or anti-actin antibody at 1:200, and with complementary HRP (Horseradish Peroxidase)-conjugated antibodies (Jackson, USA) as second antibodies at 1:5000. When inhibitors were tested, Actinomycin D 5 μg/ml or cyclohexamide 50 μg/ml was added 6 h prior to lysis and blotted as described above. For detection of the cell death pathway, cells (control and Rev-expressing) were lysed, as described above, at 16 h post-cell arrest. The lysate obtained was subjected to an SDS PAGE, transferred to nitrocellulose membrane and immunoblotted with the following antibodies: anti-active caspase 3 1:200 (marker for apoptosis [24]), antitumor necrosis factor α (TNF α) 1:200 (marker for necrosis [25]), or apg16 1:750 (marker for autophagy [26]). Then the membrane was incubated with the complementary HRP-conjugated antibodies at 1:5000 (Jackson, USA) as secondary antibodies. Quantitative estimation of the bands was performed by Image Gauge V3.46 software (Fujifilm).

Synthesis and purification of peptides

Peptides (see Table 1) were synthesized on an Applied Biosystems (ABI) 433A peptide synthesizer. The peptides were labeled with Trp at their N terminus for UV spectroscopy. Peptides purification was performed on a Gilson HPLC using a reverse-phase C8 semi-preparative column (ACE, advanced chromatography technologies, USA) with a gradient from 5 to 60% acetonitrile in water (both containing 0.001% v/v trifluoroacetic acid). Peptides concentrations were determined using a UV spectrophotometer (Shimadzu Kyoto, Japan) as described previously [27].

Effect of the peptides on cell viability using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Following incubation of the cells with 5 μg/ml Aphidicolin, the medium was removed at different times after treatment and the cells were further incubated in Earl's solution containing 0.3 mg/ml MTT for 1 h. Subsequently, the solution was removed and the cells were dissolved in 100 μl DMSO for 10 min at room temperature. The DMSO-solubilized cells were transferred to a 96-well ELISA plate, and OD values were monitored at a wavelength of 570 nm. When peptides were used, 100 μM of the indicated peptide was added with the Aphidicolin at zero time.

Results

To study Rev expression in a controlled experimental system, several human derived cell lines were transfected with a Rev expression vector and cell division was arrested by the addition of Aphidicolin (Fig. 1a). The viability of the Rev-expressing cells was greatly affected by the addition of

Table 1 List of peptides used

Peptide name	Amino acid residues	Sequence	Reference
Rev–NLS	W + Rev 35–50	W-RQARRNRRRRWRERQR	[31]
Pen-Rev–NES	W + Pen + Rev 75–84	W-Pen-LQLPPLERLTL	[31]
Pen-Rev M10-NES	W + Pen + Rev M10 75–84	W-Pen-LQLPPDLRLTL	[32]
Pen	Ant 43–58	RQIKIWFQNRRMKWKK	[33]

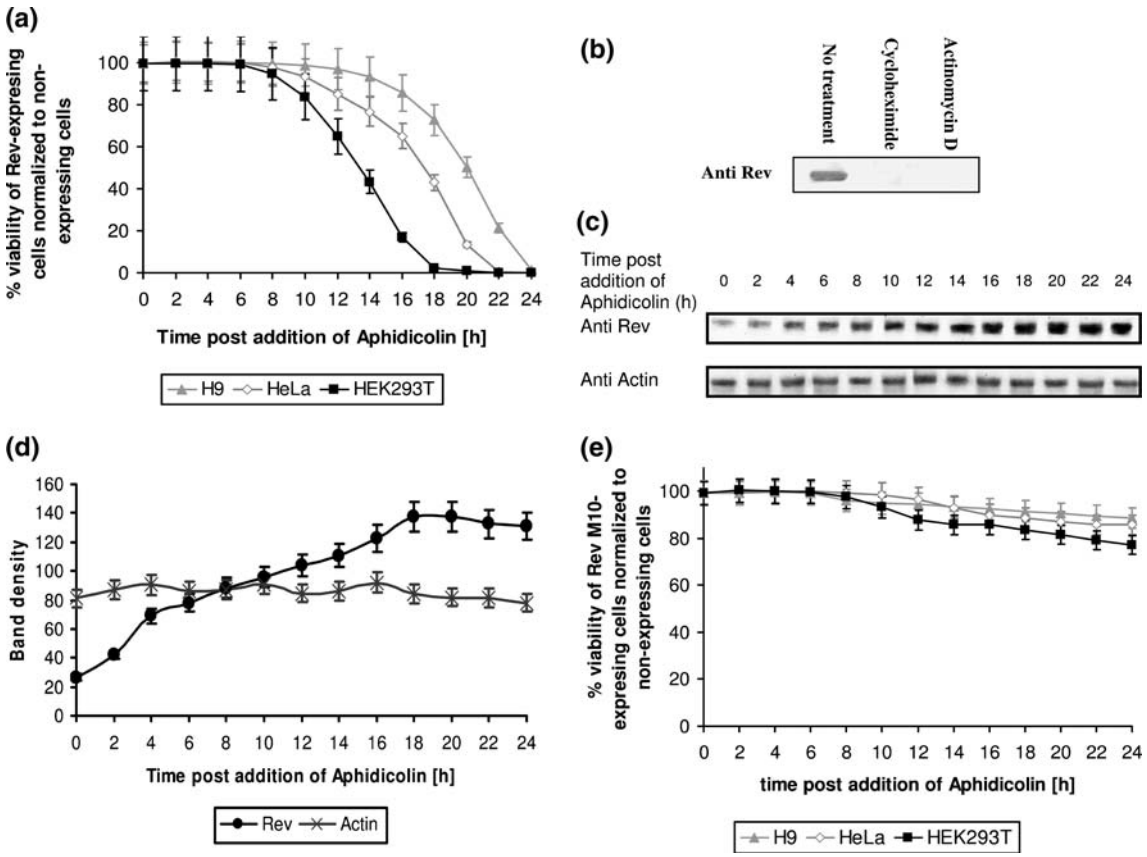

Fig. 1 Induction of death in Rev-expressing cultured cells arrested by Aphidicolin. **a** Cells were transfected with a full-length WT Rev-expressing vector and their division was arrested by Aphidicolin. Cell viability was monitored by the MTT assay. **b** Appearance of Rev and inhibition of Rev synthesis by Cyclohexamide and Actinomycin D as was detected by Western blot analysis of transfected cells. **c** HEK293T Rev-expressing cells were treated by Aphidicolin and the amount of Rev and actin was estimated by Western blot. **d** Quantitative estimation of the bands presented in (**c**) by Image Gauge V3.46 software (Fujifilm). **e** As in (**a**), but cells were transfected with a vector bearing the Rev M10 mutated gene. All other experimental details as described in "Materials and methods"

Aphidicolin. The results in Fig. 1a show that cells transfected with the Rev expression vector died between 18 and 24 h following the addition of Aphidicolin as compared to nontransfected cells that remained, essentially, fully alive under the same experimental conditions. Furthermore, cell death was not observed in dividing Rev-expressing cells (not shown and see "Discussion"). The Rev-induced cell death was observed in cells growing either as monolayer or in suspension (Fig. 1a and see "Discussion"). Promotion of cell division arrest did not affect the appearance of Rev, whose synthesis could be blocked, as expected, by either Cyclohexamide or Actinomycin D (Fig. 1b). As anticipated, accumulation of Rev in Aphidicolin-treated cells increased in a time-dependent manner (Fig. 1c, d) and was in a direct correlation to the degree of the observed cell death (Fig. 1a). Surprisingly, very little or almost no cell death was promoted in Aphidicolin-treated cells, expressing the M10 Rev mutant protein (Fig. 1e), which, as opposed to the WT Rev, remains intranuclear [28].

The experiments described in Fig. 2a show that addition of a cell permeable peptide bearing the Rev NES sequence (pen-NES, see Table 1) promoted, similar to the Rev protein, high degree of cell death. On the other hand, a peptide bearing the sequence of a mutated NES

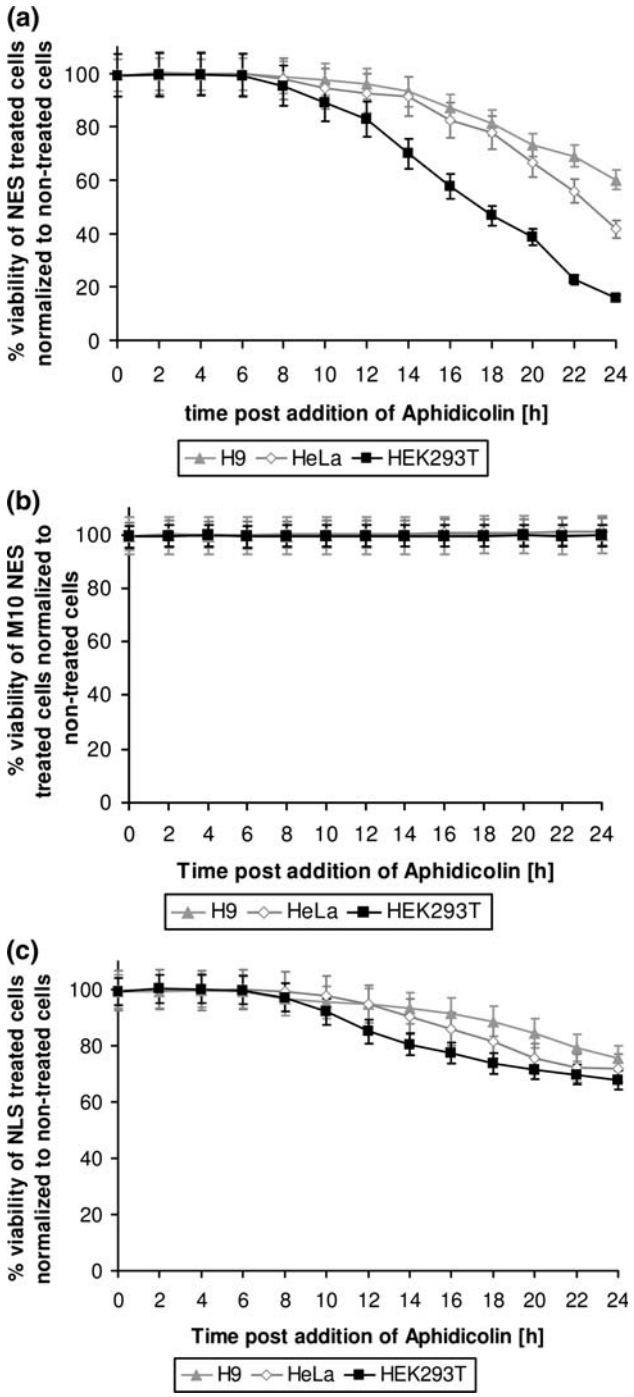

Fig. 2 The effect of peptides bearing the Rev ARM and NES sequences on the viability of Aphidicolin-arrested cells. Peptides bearing the NES (**a**) M10 NES (**b**) and the NLS (ARM) (**c**) sequences were added and cell viability was determined as described in "Materials and methods"

(residues 75–84 of the M10 Rev) had, similar to expressing of the M10 Rev (Fig. 1e), very little effect on the viability of the various cell lines studied (Fig. 2b). Similarly, a peptide bearing the Rev ARM sequence, which mediates the interaction with importin β [4–7], had

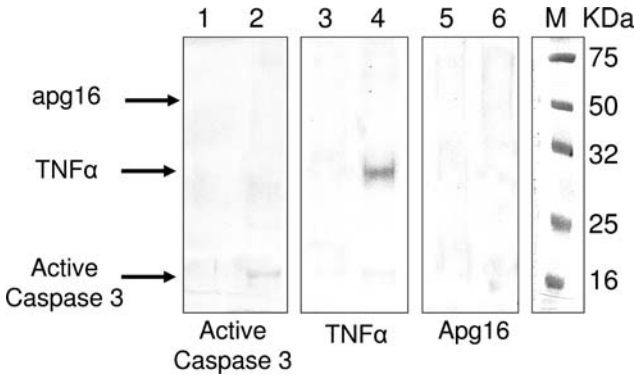

Fig. 3 Rev-induced necrotic death in arrested cultured cell. 293T cells (*lanes 1, 3, and 5*) and Rev-expressing 293T cells (*lanes 2, 4, and 6*) were arrested by incubation with Aphidicolin as described in "Materials and methods." Cells lysate was obtained 16 h post-cell arrest, was SDS PAGE and immunoblotted with anti-active caspase 3 (*lanes 1 and 2*), anti-TNF α (*lanes 3 and 4*), and anti-apg16 (*lanes 5 and 6*). *M* indicate molecular weight markers

a very little effect on cell viability when compared to the effect of the NES peptide (compare Fig. 2c to a).

It appears that the Rev-induced cells death is mostly a caspase-independent pathway, as can be inferred from the Western blot that shows the appearance of TNF α (necrosis marker [25]) (Fig. 3). A minor band of active caspase 3 (apoptosis marker [24]) can also be observed (Fig. 3). In contrast, no autophagy cell death is observed following Western blot using the anti-apg16 (autophagy marker [26]) (Fig. 3).

Discussion

During recent years, several attempts, including ours, have been made to express the WT full-length HIV-1 Rev protein in human cultured cells. These efforts failed so far. Our results explain the reason for this failure. Here we show that upon reaching confluency, Rev-expressing cells die within 20–24 h (see scheme in Fig. 4). Death of Rev-expressing cells was also observed by us when the division of these cells was arrested. This may indicate that in nondividing cells the expressed Rev protein is accumulated until reaching a certain concentration in which it becomes toxic.

Based on the results obtained, we assume that the observed Rev-induced cell killing can be attributed to the saturation of the CRM1 receptor by the relatively high concentration of the accumulated intranuclear Rev. This is supported by the results showing that similar to the WT Rev protein, also a peptide bearing the WT Rev NES-induced death of the nondividing cells. On the other hand, our results show that the expression of the Rev M10, mutant or treatment of non-Rev-expressing cultured cells with a peptide bearing a mutated NES, which lacks the

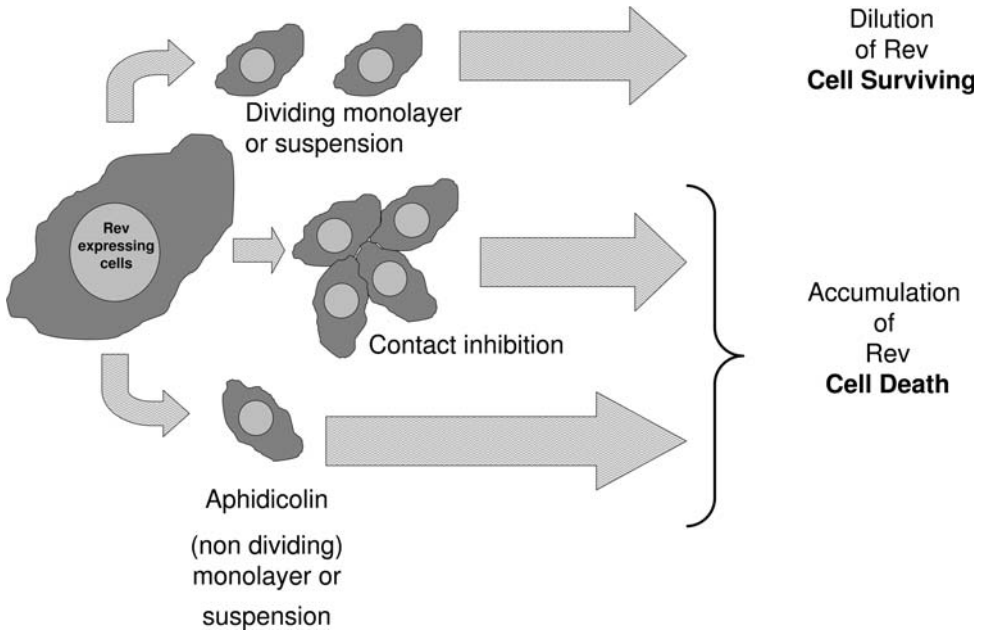

Fig. 4 A scheme describing the experimental systems used and the results obtained in this study (see text for details)

ability to bind CRM1, did not result in death of the non-dividing cells. The specificity of the NES effect could be inferred from the results showing that the Rev NLS peptide, which does not interact with the CRM1 receptor, did not induce cell death. This assumption is supported by the previous observation showing expression of the full-length Rev M10 protein in human cultured cells [28].

The Rev associated cell death was found to be correlated with the appearance of TNF α, which is known as a marker of necrotic cell death [25]. However, this does not necessarily indicate a process of necrosis cell death. TNF α may induce both caspase-dependent (apoptotic cell death [29, 30]) and caspase-independent cell death (necrosis cell death [25]). Although it appears that most of the Rev-induced cell death is due to caspase-independent mechanism, some degree of caspase-dependent cell death could occur as shown by the presence of low levels of active caspase 3. In summary, our results show that in order to obtain a stable Rev-expressing cell line and avoid cell death, cells should be prevented from either reaching confluency or cell division arrest.

Acknowledgments This study was supported by the Israeli Science Foundation (AL) and by a starting grant from the European Research Council (ERC) (to AF).

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