Nucleocytoplasmic transport in human astrocytes: decreased nuclear uptake of the HIV Rev shuttle protein

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SUMMARY

Astrocytes are cellular targets for the human immunodeficiency virus (HIV) that limit virus production, owing, at least in part, to the diminished functionality of the viral post-transcriptional stimulatory factor Rev. To understand the trafficking process in astrocytes, we compared nucleocytoplasmic transport of Rev and various proteins with well-characterized nucleocytoplasmic transport features in human astrocytes and control cells (HeLa). Localization and trafficking characteristics of several cellular and viral proteins, as well as nuclear trafficking of classical peptide signals upon microinjection were similar in both cell types, indicating maintenance of general features of nucleocytoplasmic transport in astrocytes. Quantification of fluorescence in living cells expressing Rev fused to green fluorescent protein (GFP) indicated a strong shift in intracellular distribution of Rev in astrocytes, with 50-70% of Rev in the cytoplasm, whereas the cytoplasmic proportion of Rev in HeLa cells is around 10%. The dynamics of nucleocytoplasmic trafficking of Rev were compared in astrocytes and Rev-permissive cells by monitoring migration of Rev-GFP in cell fusions using highly sensitive time-lapse imaging. Nuclear uptake of Rev was dramatically retarded in homo-polykaryons of astrocytes compared with control cells. Diminished nuclear uptake of Rev was also observed in hetero-polykaryons of Rev-permissive cells and astrocytes. These results indicate that astrocytes contain a cytoplasmic activity that interferes with nuclear uptake of Rev. Our studies suggest a model in which Rev is prevented from functioning efficiently in astrocytes by specific alterations of its nucleocytoplasmic trafficking properties.

Movies available on-line:
http://www.biologists.com/JCS/movies/jcs1709.html

Key words: Astrocytes, Shuttling, HIV, Rev, PKI, Importin β, Exportin 1, B23, Ran/TC4

INTRODUCTION

In eukaryotic cells, complex mechanisms have evolved for controlled transport of RNAs and proteins between cytoplasmic and nuclear compartments (for reviews, see Gorlich and Kutay, 1999; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998). Nucleocytoplasmic exchange is mediated by transport receptors. These interact with both specific transport signals in cargo molecules and nucleoporins of pore complexes (NPCs) in the nuclear envelope. The complexes translocate through NPCs in an energy-dependent process. Transport receptors bind to a small GTPase called Ran, which exists predominantly in the GTP bound form (RanGTP) in the nucleus and as RanGDP in the cytoplasm. This asymmetric distribution of RanGTP/GDP determines the directionality of transport, and hydrolysis of RanGTP to RanGDP provides the transport cycle with energy. A number of transport pathways have been recently characterized. In humans, the importin β superfamily of transport receptors comprises at least 21 members, which recognize various nuclear transport signals and cargo molecules. Among the best characterized members of the importin β superfamily are the nuclear import receptor importin β, which binds its cargo alone or via the importin α adapter, and the export receptor exportin 1 (crm1), which interacts with nuclear proteins containing leucine-rich transport signals. These factors mediate nucleocytoplasmic transport not only of cellular but also of numerous viral proteins and thus are essential cellular factors for replication of many viruses (reviewed by Whittaker and Helenius, 1998).

The Rev protein of the human immunodeficiency virus (HIV) is a prototypical nucleocytoplasmic shuttle protein. Rev has signals for both nuclear import and export that interact with importin β and exportin 1 transport receptors (for reviews, see Cullen, 1998a; Kjems and Askjaer, 2000; Pavlakis and Stauber, 1998; Pollard and Malim, 1998). Rev selectively stimulates production of HIV structural proteins by binding the Rev response element (RRE) in intron-containing viral mRNAs and promoting their export from the nucleus to the cytoplasm. Rev is a small (116 amino acids, 18 kDa) phosphoprotein, found mainly in the nuclei of Rev-expressing cells, where it accumulates in the nucleoli. The N-terminal domain of Rev

contains an arginine-rich motif (ARM; aa 35-50) involved in nuclear localization and RNA binding of Rev. Sequences flanking the ARM are required for Rev oligomerization. The nuclear localization signal (NLS) in the ARM of Rev was mapped by its capacity to mediate nuclear accumulation of a heterologous protein (Bohnlein et al., 1991; Cochrane et al., 1990; Kubota et al., 1989; Venkatesh et al., 1990). The NLS of Rev differs from lysine-rich 'classical' NLS elements in SV-40 T-antigen or nucleoplasmin, because it is arginine-rich and does not require the importin α adapter to interact with importin β in *in vitro* assays (Henderson and Percipalle, 1997; Truant and Cullen, 1999). Mutations within the NLS (Berger et al., 1991; Hammerschmid et al., 1994; Malim et al., 1989) as well as in sequences flanking the ARM (Hope et al., 1990; Malim et al., 1989; Stauber et al., 1998a; Szilvay et al., 1997) disrupt typical nuclear/nucleolar localization of Rev, suggesting that sequences outside the NLS influence nuclear accumulation. The C-terminal functional domain of Rev contains a leucine-rich nuclear export signal (NES; aa 75-83) (Fischer et al., 1995; Wen et al., 1995), which interacts with exportin 1 (Fornerod et al., 1997a) for export of the Rev-RNA complex to the cytoplasm. Both functional domains of Rev have been shown to interact with various cellular factors (reviewed by Kjems and Askjaer, 2000).

Astrocytes are target cells for HIV that *in vivo* rarely show signs of active virus production and harbor mainly nonstructural components of HIV (reviewed by Brack-Werner, 1999). In culture, production of HIV by infected astrocytes is dramatically reduced compared with T cells or microglial cells (Brack-Werner et al., 1992; McCarthy et al., 1998). Several mechanisms have been proposed to limit HIV-1 replication in astrocytes, including restrictions of viral entry and viral gene-expression (reviewed by Brack-Werner and Bell, 1999). We have demonstrated in extensive studies with tumor-derived and primary astrocytic cell cultures that functionality of Rev is severely diminished in astrocytes (Ludwig et al., 1999; Neumann et al., 1995). Astrocytes display altered localization of Rev, with Rev distributed in both nuclear and cytoplasmic compartments. Inhibition of nuclear export of Rev causes Rev to accumulate in the nuclei of astrocytes (Ludwig et al., 1999), indicating that Rev is capable of nuclear translocation in these cells.

Here, we compare nucleocytoplasmic transport of Rev and various proteins with well-characterized transport features in astrocytes and HeLa cells. Our studies indicate that nucleocytoplasmic transport processes in astrocytes resemble those in HeLa cells in many respects. However, astrocytes have biological properties that appear to specifically disrupt nucleocytoplasmic distribution of Rev and that inhibit nuclear uptake of Rev during shuttling. These results suggest that Rev is prevented from functioning efficiently in certain cell types by specific modulation of its nucleocytoplasmic trafficking properties.

MATERIALS AND METHODS

Cell lines and transfections

HeLa cells are a human cervical adenocarcinoma cell line with epithelial morphology. Glioblastoma/astrocytoma cell lines U87MG, U138MG and U373MG were obtained from the American Type Culture Collection (ATCC HTB-14, -16, -17, Rockville, MD). 85HG66 is a human astrocytoma cell line that has been described previously (Stavrou et al., 1987; Brack-Werner et al., 1992). Cells were kept under standard cell culture conditions using Dulbecco's Modified Eagle Medium with 10% FCS and 2 mM Glutamax I (Life Technologies, Karlsruhe, Germany). For microscopy and imaging purposes, cells were cultured in medium without phenol red. Cells were transfected with plasmid DNA purified by ion exchange chromatography columns (Qiagen, Hilden, Germany) using the calcium phosphate coprecipitation technique (Ludwig et al., 1999) (CellPhect-kit, Pharmacia, Germany). Transfection mixtures typically contained 1-3 μg of the plasmid expressing the gene of interest, adjusted to 17 μg total DNA with a Bluescript-derivative (pBSPL) used as inert carrier plasmid. Transfection efficiencies were estimated by visual inspection of GFP-expressing cells and lay between 10 and 40% transfected cells, depending on the cell type.

Primary fetal astrocyte cultures (kindly provided by Francesca Aloisi, Istituto Superiore di Sanità, Rome, Italy) were prepared from myencephalon-mesencephalon (H4/96III) of a 9-week-old fetus and cultured as described previously (Aloisi et al., 1992; Ludwig et al., 1999). For transfection experiments, fourth-passage cells were seeded on poly-L-lysine-coated glass bottom dishes at a density of 3×10^5 cells per 35 mm diameter dish and grown at 37°C in a 5% CO_2 humidified atmosphere.

Constructs

The constructs pCsRevsg143 and pCsRevM10BLsg143 (Ludwig et al., 1999) and the HIV-1 Tat-GFP expression construct pTat-GFP (Stauber and Pavlakis, 1998) have been described previously. The GFP expression constructs pFred25 and pFred143 were used for creating fusion proteins; both contain GFP mutants with fluorescence signal strength approximately 50- and 100-fold that of wild-type GFP (Stauber et al., 1998b). Proteins of interest were fused to the N-terminus of GFP by using a unique NheI-site located between the second and third codon of GFP for insertion of appropriate cDNAs. A GlyAlaGly hinge region separated the proteins of interest from the GFP domain. HIV-1 *nef* was amplified from pNL1.5.7 (Schwartz et al., 1990) and cloned into the BssHII/NheI site of pFred25. cDNAs for B23 (nucleophosmin; Chan et al., 1989) (GenBank accession number M23613) and for Ran/TC4 (Drivas et al., 1990; GenBank accession number M31469) were PCR-amplified from reverse-transcribed HeLa RNA, and cloned into pFred25 to yield pCB23sg25 and pCRansg25, respectively. The sequence for the small thermostable protein kinase inhibitor PKI-α (Olsen and Uhler, 1991; GenBank accession number S76965) was amplified from reverse-transcribed RNA of the astrocytoma cell line U138MG and cloned into pFred143. Human exportin 1 (hCRM1) was amplified from a cDNA clone kindly provided by Maarten Fornerod (pT7-hCRM1, GenBank accession number Y08614; Fornerod et al., 1997b) and cloned into pFred143 as described.

Microscopy setup and fluorescence quantification

Epifluorescence microscopy was performed using an inverted research microscope (Zeiss Axiovert 135TV, Carl Zeiss Göttingen, Germany). Routine microscopy and polykaryon assay observation was performed using long working distance Achrostigmat objectives (10×, 20× and 32× magnification, phase 1). High-resolution localization studies were performed with a long working distance Achroplan 40× water immersion objective (phase 2). For this purpose, cells were grown on 35 mm glass bottom dishes (Mattek Corp., Ashland, MA) to allow for the high numerical aperture of the Achroplan objective. Transmitted light images were usually taken as phase-contrast or as differential interference contrast (Nomarski) images. Culture dishes were put on a heated stage within a mounted controlled-environment chamber (Carl Zeiss, Göttingen, Germany), allowing the continuous cultivation of the cells at 37°C with 5% CO_2 and high humidity during imaging. Excitation light was provided by a dimmable AttoArc power supply (Carl Zeiss, Oberkochen, Germany) driving a 100 W Mercury bulb. An electronically controlled filterwheel (Ludl Electronic Products, New York, NY) contained a set of narrow bandwidth excitation filters

with different wavelength maxima (Set 83,000, Chroma Technologies, Brattleboro, VT) to allow computer-controlled change of illumination. The following excitation wavelengths were used for time-lapse studies and analysis of microinjections: GFP/Alexa-Green: 493 nm and Hoechst 33342: 357 nm. A three-bandpass beamsplitter and a multi-bandpass emission filter allowed automated detection of all emission wavelengths. Analysis of immunofluorescence results obtained with cyanine 3-labeled antibody was performed using the Zeiss 14 filterset with two excitation maxima at 510 and 560 nm, and a longpass emission filter (590 nm and over). For time-lapse studies, images were taken automatically every few minutes by a computer-controlled, high resolution slow scan CCD camera (Quantix, Photometrics, Tucson, AZ; Kodak KAF1400 grade 1 chip). For quantification purposes, care was taken to integrate individual frames under subsaturating conditions (i.e. no pixel reaching an intensity value of 4095). An area near the quantified cells was chosen as background field to correct for intensity gradients due to uneven illumination.

For time-lapse series, frames were taken with identical integration times, usually 1-3 seconds per frame at a 2×2 binning using an electronic gain of 2. Images were stored and analyzed on an Apple Macintosh PowerPC using IPLab Spectrum 3.2.4 software (Scanalytics, Vienna, VA). Frame series were put together to create QuickTime movies of the events. The 12-bit gray-scale images were normalized, the contrast adjusted and the images exported in TIFF or PICT format either as 8-bit gray-scale or as 24-bit color images (for a more detailed description of this procedure, see Lee et al., 1999). Images were arranged into panels using standard graphics programs (Adobe PhotoShop 5, Adobe Illustrator 8, Adobe Systems, USA; Graphic Converter 3.9.1, Lemkesoft, Ulm, Germany).

In some figures (indicated where applicable), images were recorded on regular photographic diapositive slides (Fuji Provia 1600), scanned using a filmscanner (Polaroid Sprintscan 35, Polaroid, USA) and exported into Adobe PhotoShop for presentation purposes.

Immunofluorescence and antibodies

Immunofluorescence was essentially performed as described previously (Neumann et al., 1995). Human importin β was detected with an affinity purified polyclonal serum raised in rabbits (kindly provided by Dirk Görlich, University of Heidelberg, Germany; Gorlich et al., 1995) at a dilution of 1:50. Human exportin 1 was detected using a rabbit polyclonal antiserum at a dilution of 1:200 (kindly provided by Maarten Fornerod, EMBL, Heidelberg, Germany). Primary antibody binding was detected with a goat polyclonal anti-rabbit antiserum coupled to cyanine 3 (Dianova, Hamburg, Germany) and diluted 1:200.

Microinjections

Bovine serum albumin (Sigma, Germany) was coupled to fluorescent Alexa-Dyes with excitation maxima at 488 nm (green emission) and 568 nm (red emission) according to manufacturers instructions (Molecular Probes, The Netherlands). Alexa-red labelled BSA was coupled to peptides representing either the nuclear localization signal (NLS) of the large-T-antigen of SV-40 (CGGGPKKKRKVED), the nuclear export signal (NES) of the HIV-1 Rev protein (CGGGLQLPPLERLTLD) or the Rev-ARM motif (Rev-aa positions 35-51; CGGRQARRNRRRRWRERQRQ) essentially as described previously (Fischer et al., 1995). Coupling of the Rev-ARM frequently led to precipitation of the reagents, destroying the coupling reaction. The chemically reactive cysteine residue was separated from the signal sequences by two to three glycine residues serving as a flexible hinge. Approximately 10-20 peptide molecules were bound to one BSA molecule as estimated by polyacrylamide gel electrophoresis.

Two additional conjugates containing peptides encompassing the ARM motif of Rev (Rev-aa position 35-43 RQARRNRRRC) and of Tat (Tat-aa position 48-61, GRKKRRQRRRAHQN), coupled C-terminally to BSA were kindly provided by Prof. Abraham Loyter, Hebrew University, Israel.

Microinjections were performed using an Eppendorf microinjector (Eppendorf, Hamburg, Germany) and with a CompInject AIS2 automated microinjection system (Cell Biology Trading, Hamburg, Germany) mounted on an inverted Zeiss 35 microscope. Cells were seeded on glass-bottom dishes at semi-confluence. A mixture of Alexa-green labelled BSA and Alexa-red labelled BSA coupled to signal peptides (1 mg/ml each) was injected into the cytoplasmic or nuclear compartment. One hour after microinjection, cells were fixed for 20 minutes with 3.7% buffered formaldehyde and nuclei counterstained by a 10 minute incubation with 2 μg/ml Hoechst 33342 dye. Images of blue, green and red channels were taken using the imaging setup described above. Red channel images served as control to ensure that the injection compartment remained intact after injection.

For PEG-fusion assays, in some cases donor cells were microinjected with purified Rev-GFP expression plasmid (10 ng/μl in PBS).

Polykaryon assay

Cells were plated in 60 mm diameter dishes at 30% confluency and transfected as described. After 8-12 hours, cells were inspected by fluorescence microscopy at 488 nm excitation wavelength for expression of the GFP fusion proteins. Usually 10-40% of the cells showed strong fluorescence. Non-transfected cells were added to the dish at a tenfold excess (ratio of 10:1) 18-24 hours after transfection to achieve confluency the next day. The final number of cells was 1×10^6 cells (U138MG) or 1.5×10^6 cells (HeLa and 85HG66) per 60 mm diameter dish. The confluent cell layer was visually inspected for even distribution of green fluorescent cells surrounded by non-transfected (i.e. non-fluorescent) cells. Approximately 60% of fluorescent cells appeared as doublets, indicating a recent cell division event. Cells were washed once with prewarmed PBS and fused for 2 (HeLa) or 3 (astrocytes) minutes by addition of a prewarmed 50% solution of PEG 4000 in PBS (Boehringer-Mannheim, Germany). PEG was removed thoroughly by four washes with prewarmed medium and the cells were refed with prewarmed medium without phenol red. Cells were observed under phase-contrast and fluorescent illumination, and quickly scanned for likely fusion events involving few fluorescent donor cells surrounded by nontransfected acceptor cells, before capturing images. Image capture started 5-10 minutes after initiation of fusion. In some experiments, cells were treated with 25 μg/ml cycloheximide (Sigma, Deisenhofen, Germany) to block new protein synthesis, 2 hours before fusion and for the duration of the experiment. In some experiments, nuclei were counterstained before fusion with 2 μg/ml of the live cell DNA stain Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) for 10 minutes.

Throughout the text, we refer to cell fusions of the same type (e.g. HeLa×HeLa) as 'homo-polykaryons' and fusions of cells of different types (e.g. HeLa×85HG66) as 'hetero-polykaryons'. Hetero-polykaryon assays were performed with selected populations of Rev-GFP expressing donor cells. These were obtained either by FACS sorting of transfected cells or by directly microinjecting Rev-GFP expression plasmids into nuclei of donor cells prior to addition of acceptor cells. Staining with Hoechst 33342 dye allowed discrimination of astrocytoma- and HeLa-cell-derived nuclei. This confirmed the presence of an excess of acceptor nuclei over donor nuclei in hetero-polykaryons.

Several criteria were applied to the selection of cell fusions for quantitative evaluation. First, cell fusions were selected containing at least 2 and less than 20 nuclei. If fusions were larger, quantification of acceptor nuclei became difficult because extreme dilution of the fluorescence signal made significant measurement over background difficult. Furthermore, only nuclei that remained clearly separated from neighboring nuclei during the imaging period were analyzed, to avoid overlay of signal. Second, imaging was performed under homogeneous and stable lighting conditions with defined exposure times below saturation levels of the detector. Third, cell fusions were kept at ideal environmental conditions (37°C, 5% CO_2, humidified

atmosphere) during imaging. Shuttling rates were reduced dramatically when imaging was performed on a microscopic stage at room temperature (data not shown). Finally, illumination times were kept at a minimum to prevent bleaching of the signals, especially when images were taken in rapid succession.

Statistical analysis

Statistical analysis of data was carried out with GraphPad PRISM, Version 2.0, (GraphPad Software, Inc., San Diego, CA). Significance between data sets was determined by calculating two-tail P-values, using the Mann-Whitney U test.

RESULTS

Steady-state accumulation of Rev in the cytoplasm of astrocytes

Previously, we showed that HIV-1 Rev localizes in the cytoplasm of human astrocytes, in contrast to the dominantly nuclear/nucleolar localization of Rev typical for cell types that support efficient Rev function (Ludwig et al., 1999; Neumann et al., 1995). To obtain a measure of this shift in intracellular distribution of Rev in astrocytes, we established conditions for quantification of Rev-GFP fluorescence in nuclear and cytoplasmic compartments of living cells. Fig. 1A shows typical Rev-GFP localization in HeLa and U138MG astrocytoma cells. Total and nuclear fluorescence intensities were quantified for single cells, corrected for background fluorescence and the proportion of cytoplasmic fluorescence calculated (Table 1). Intracellular distribution of Rev was assessed for primary fetal astrocytes (PFA), two well-characterized human astrocytoma cell lines U138MG and 85HG66, and for the Rev-permissive cell line HeLa. In addition, quantifications were performed for three nuclear control proteins: the export-deficient Rev-mutant M10BL (RevM10BL-GFP), HIV-Tat (Tat-GFP) and B23 (B23-GFP; Zirwes et al., 1997), a nonviral nucleolar protein.

Fig. 1B shows the percentage of cytoplasmic fluorescence obtained with the GFP-tagged proteins for each cell line (9-38 cells per construct). Astrocytic cells (PFA, 85HG66 and U138MG) contained, on average, 47-66% of total Rev-GFP in the cytoplasm at steady-state, whereas HeLa cells contained only 10% cytoplasmic Rev-GFP. This difference is highly significant (P<0.0001; Mann-Whitney t-test for non-parametrically distributed values). By contrast, the nuclear/nucleolar control proteins RevM10BL-GFP and B23-GFP are predominantly nuclear in both astrocytes and HeLa cells, with less than 10% in the cytoplasmic compartment.

Tat is another viral regulatory protein with an ARM motif, similar to Rev. Cytoplasmic accumulation of Tat-GFP was significantly increased in U138MG (37%, P=0.0033) and PFA (37%, P=0.0294), compared with HeLa cells (23%), but not in astrocytoma cell line 85HG66 (25%, P=0.3538). Compared with Rev-GFP, levels of cytoplasmic accumulation of Tat-GFP were significantly lower in 85HG66 (52% versus 25%, P<0.0001) and U138MG (66% versus 37%, P<0.0001) but not in PFA (47% versus 37%, P=0.2539).

These results indicate that Rev consistently accumulates in the cytoplasmic compartment of cultured astrocytes of various origins, suggesting that localization behavior of Rev is generally altered in astrocyte cultures.

Similar localization of other proteins with distinct compartmentalization properties in astrocytes and HeLa cells

To learn more about nucleocytoplasmic targeting properties of astrocytes, we investigated the compartmentalization behavior of the following cellular proteins with defined nucleocytoplasmic transport characteristics: Ran-TC4, a small nuclear GTPase essential for nucleocytoplasmic protein transport (Ren et al., 1993); exportin-1, a nuclear export receptor for proteins with a Rev-like NES (Bogerd et al., 1998; Fornerod et al., 1997a); PKI-α, which mediates translocation of catalytic subunits of cyclic AMP-dependent protein kinase (PKA) from the nucleus to the cytoplasm by means of a Rev-like NES (Wen et al., 1995) and the nucleolar protein B23 (Borer et al., 1989). In addition, we tested the early HIV-1 protein Nef, an important marker for HIV infection of astrocytes (Brack-Werner and Bell, 1999; Brack-Werner et al., 1992; Kohleisen et al., 1999; Ranki et al., 1995; Tornatore et al., 1994). Nef has been reported to localize to the plasma membrane, the perinuclear trans-Golgi network (Greenberg et al., 1997; Kaminchik et al., 1994) and to nuclei (Kohleisen et al., 1992; Murti et al., 1993; Ovod et al., 1992). In reference to previously published results (Ludwig et al., 1999), we included localization results for Rev, the export deficient RevM10BL and HIV Tat-GFP.

Intracellular localization patterns of proteins expressed with GFP-tags were compared in primary fetal astrocytes, two

Table 1. Calculation of cytoplasmic fluorescence in rev-GFP-expressing cells

Cell line	Segment	Area (pixel)	Fluorescent signal				Cytoplasmic fluorescence (%)
			Measured	Background*	Corrected	Proportion (%)	
HeLa							
Cell 1	Entire cell	4,372	1,699,811	700,224	999,587	100.0	
	Nucleus	1,631	1,234,512	261,223	973,289	97.4	2.6
Cell 2	Entire cell	5,867	1 544,845	939,665	605,180	100.0	
	Nucleus	1,734	855,753	277,719	578,034	95.5	4.5
U138MG							
	Entire cell	11,469	3,036,152	2,422,908	613,244	100.0	
	Nucleus	2,126	612,037	449,133	162,904	26.6	73.4

*Background signal = (mean signal intensity per pixel in background segment) × (area of measured segment).

Total fluorescent signal measured for each cell segment was corrected for background fluorescence. The corrected signal value of the entire cell segment was set to 100% and proportions of nuclear and cytoplasmic signals calculated.

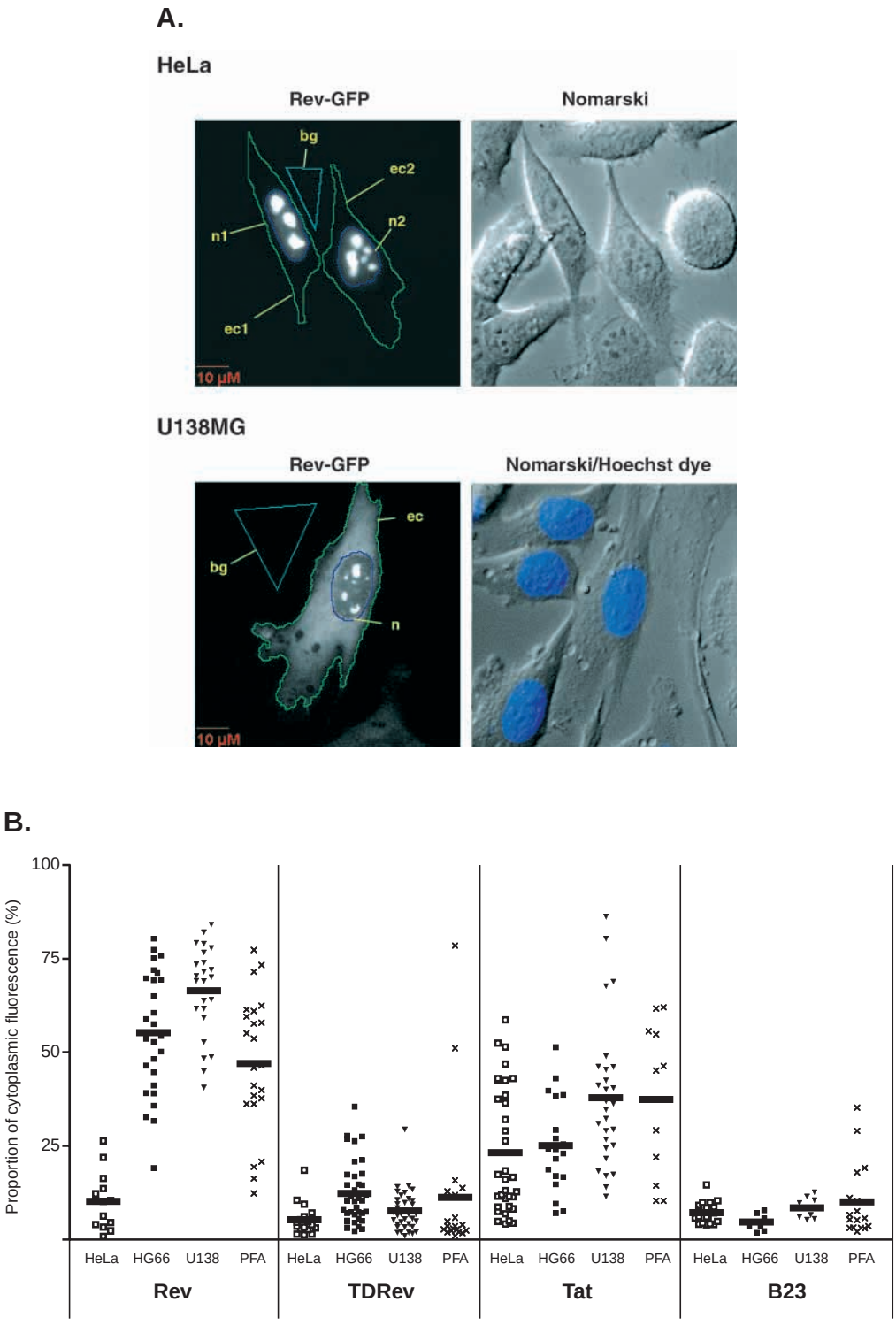

Fig. 1. Nucleocytoplasmic distribution of the HIV-1 Rev shuttle protein in astrocytes and control cells. (A) Procedure for quantification of Rev-GFP fluorescence in living cells. The quantification procedure is exemplified for typical images of a HeLa cell and an astrocyte (U138MG) expressing Rev-GFP fusion protein after transfection with pCsRevsg143 plasmid. Images were taken under non-saturating conditions of the CCD detector (see Materials and Methods). The total fluorescent signal of the entire cell (ec) and of its nuclear compartment (n) was measured. Transferring cellular outlines from Nomarski images to fluorescent images assisted delineation of cellular boundaries. In addition, delineation of nuclear boundaries in astrocytes was augmented by counterstaining cells with the nuclear dye Hoechst 33342 and transfer of segmentation information obtained with this dye to the GFP-image. Background fluorescence was determined by measuring total signal of an extracellular segment (bg) in close proximity to the measured cells and calculation of mean background signal per pixel. (B) Proportion of mean cytoplasmic fluorescence in HeLa cells (HeLa), astrocytoma cells (HG66, 85HG66; U138, U138MG) and primary fetal astrocytes (PFA) expressing Rev-GFP, RevM10BL-GFP, Tat-GFP and B23-GFP. RevM10BL is an export deficient mutant of Rev; Tat is the HIV-1 transcriptional activator; and B23 is a cellular nucleolar protein. Symbols indicate values for individual cells and horizontal bars indicate mean values for each cell line. The cytoplasmic proportion of Rev-GFP is significantly higher in astrocytes (mean of 47-66%) than in HeLa cells (mean of 10%). Intracellular distribution of RevM10BL-GFP and B23-GFP is predominantly nuclear in both cell types. Mean cytoplasmic Tat-GFP localization is 23% in HeLa, 25% in 85HG66 and 37% in both U138MG and PFA.

astrocytic cell lines (U138MG and 85HG66) and in a non-astrocytic control cell line (HeLa). As is evident from Fig. 2, localization patterns of all control proteins were similar in cells of astrocyte origin and in control cells, as opposed to the localization of Rev-GFP (Fig. 2A, bottom panel). Ran-GFP and exportin 1-GFP showed predominantly nuclear localization with a clearly visible cytoplasmic component. In addition, exportin 1-GFP showed a characteristic fluorescent ring signal around the nucleus, in agreement with its capacity to accumulate at the nuclear envelope (Fornerod et al., 1997b). B23-GFP was detected exclusively in nuclei (see also Fig. 1B), mainly in nucleoli and displayed almost identical localization to RevM10BL-GFP

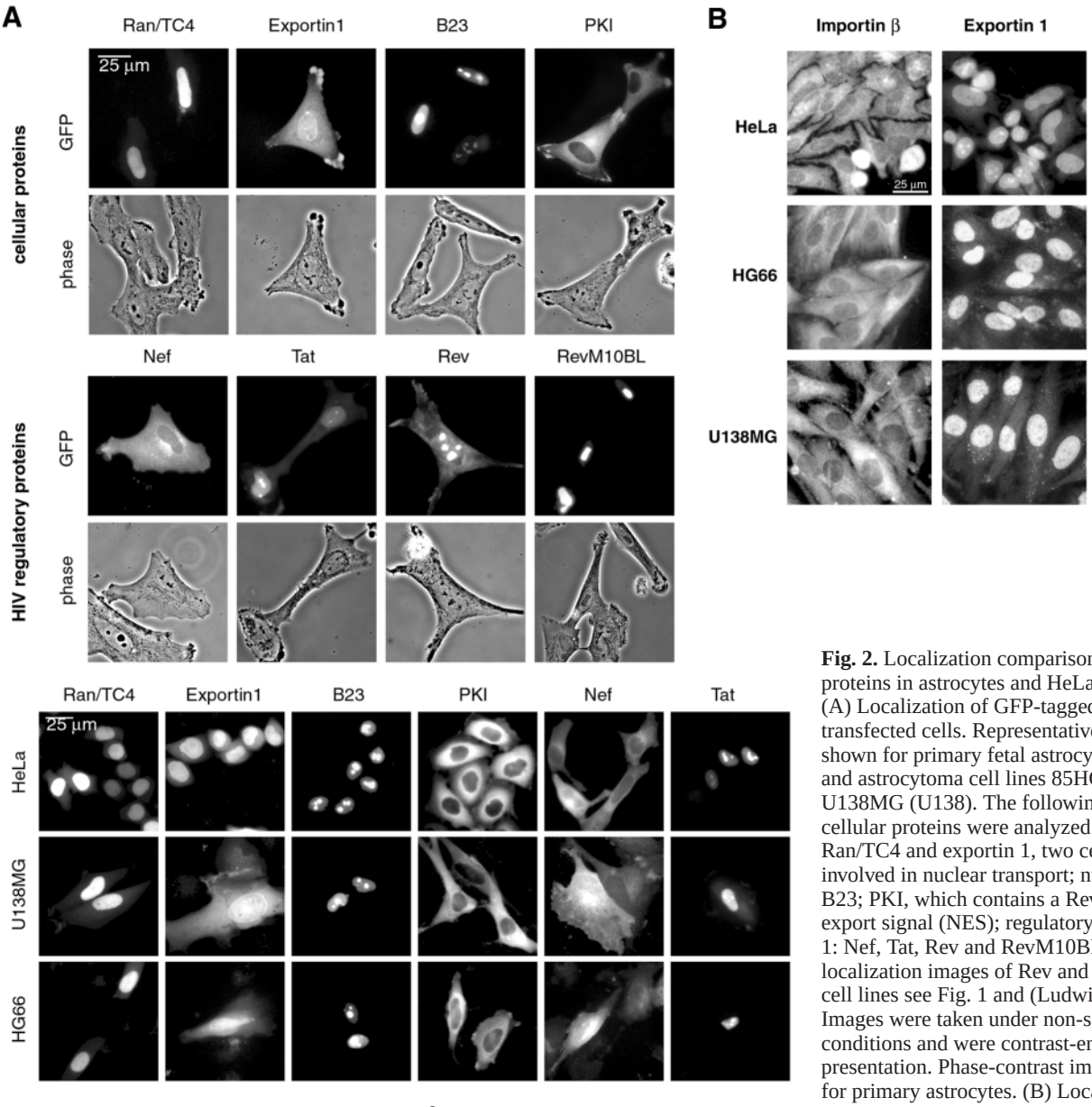

Fig. 2. Localization comparisons of various proteins in astrocytes and HeLa cells. (A) Localization of GFP-tagged proteins in transfected cells. Representative images are shown for primary fetal astrocytes, HeLa cells and astrocytoma cell lines 85HG66 (HG66) and U138MG (U138). The following GFP-tagged cellular proteins were analyzed (upper panel): Ran/TC4 and exportin 1, two cellular proteins involved in nuclear transport; nucleolar protein B23; PKI, which contains a Rev-like nuclear export signal (NES); regulatory proteins of HIV-1: Nef, Tat, Rev and RevM10BL. For localization images of Rev and RevM10BL in cell lines see Fig. 1 and (Ludwig et al., 1999). Images were taken under non-saturating conditions and were contrast-enhanced for presentation. Phase-contrast images are shown for primary astrocytes. (B) Localization of endogenous nuclear transport receptors importin β and exportin 1 was analyzed by immunofluorescence staining of HeLa cells and astrocytoma cell lines with appropriate antibodies (Materials and Methods). Images were taken at identical exposure settings for comparison.

(Fig. 2A). Tat-GFP was apparent in the nuclear/nucleolar compartment of all cell types. A weaker cytoplasmic localization can be seen in PFA and U138MG, and to a lesser degree in 85HG66 and HeLa cells (see above). By contrast, PKI-GFP was visible primarily in the cytoplasm. Finally, Nef-GFP showed typical accumulation in the perinuclear region consistent with localization in the Golgi network (see above).

To analyze localization of endogenously produced nuclear transport receptors importin β and exportin 1 in astrocytes, we performed immunofluorescence staining with appropriate polyclonal antibodies (Fig. 2B). Both proteins were clearly expressed in astrocytes and showed similar localization behavior in astrocytes and HeLa cells. Localization patterns of native exportin 1 agree with those of exogenous exportin 1-GFP (compare Fig. 2A and Fig. 2B).

In summary, astrocytes resembled HeLa cells with respect to localization of several representative proteins and expression of major nuclear transport receptors.

Correct NLS and NES-mediated protein targeting in astrocytes

To determine whether signals for nuclear import (NLS) or nuclear export (NES) are functional in astrocytes, we assessed the capacity of peptides with nuclear transport signals to mediate nuclear translocation of heterologous proteins in microinjection experiments. To this end, we chose short, well-characterized signal sequences both necessary and sufficient to direct nuclear translocation by transport receptors of the importin β superfamily and considered to represent ‘classical’ nuclear transport signals. Peptides for nuclear import contained the 7-amino acid minimal signal sequence (PKKKRKV) required for nuclear targeting of the SV-40 large T-antigen (Moore and

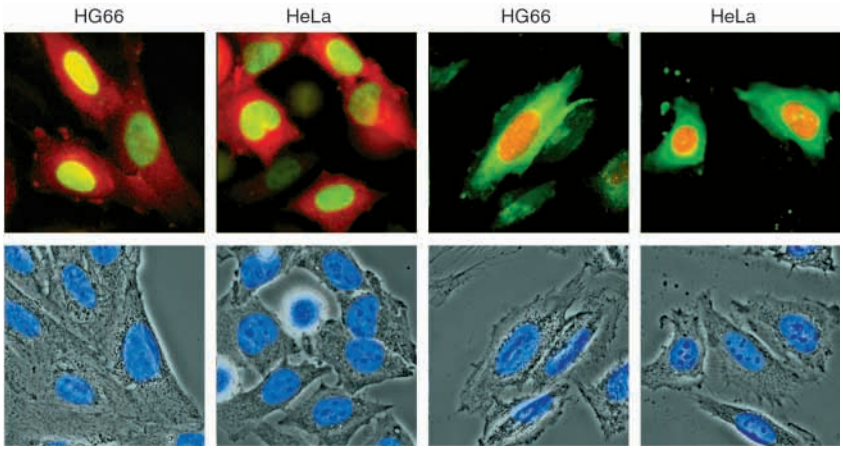

Fig. 3. Nucleocytoplasmic translocation of fluorescently labelled bovine serum albumin (BSA) conjugated with signal peptides for nuclear import (NLS) and nuclear export (NES) in microinjection assays. Peptides containing the NLS of SV-40 large T-antigen or the NES of Rev were conjugated with BSA labelled with green fluorescent dye (Alexa-green, excitation maximum 488 nm). BSA labelled with red fluorescent dye (Alexa-red, excitation maximum 568 nm) was added to injection mixtures as a control for the site of injection and preservation of nuclear integrity. 85HG66 (HG66) astrocytoma- and HeLa control-cells were seeded on glass-bottom dishes 1 day before microinjection. Injections were performed into the cytoplasmic (BSA-NLS) or nuclear (BSA-NES) compartments. Cells were fixed after a 1 hour incubation at 37°C, nuclei counterstained with Hoechst 33342 (blue) and imaged. The upper panel shows green and red channel overlays. Red signal demonstrates injections of the correct compartment and intactness of nuclear and cytoplasmic compartments in microinjected cells. Green signal demonstrates signal-mediated movement of conjugated BSA molecules. The bottom panel shows an overlay of phase-contrast and blue channel, indicating nuclei.

Blobel, 1993). Signal sequences for nuclear export contained a 12-amino acid leucine-rich segment (LQLPPLERLTLD) of the HIV-1 Rev protein defined as minimal NES (Meyer and Malim, 1994; Wen et al., 1995). Peptide-BSA conjugates (green) and an unconjugated BSA control (red) were coinjected into the nuclear or cytoplasmic compartments, cells fixed one hour post injection and nuclei counterstained with Hoechst 33342 (blue). Microinjection experiments were performed with four astrocytoma and one control cell line (HeLa). Exemplary images of injected 85HG66 astrocytoma and HeLa cells (Fig. 3) show translocation of BSA coupled with NLS- or NES-peptides to the appropriate compartment and retention of unconjugated BSA at the site of injection. Identical results were obtained with U373MG, U138MG and U87MG astrocytoma cells (data not shown). Although this technique cannot be used to determine rates of import or export in live cells, owing to the phototoxicity of the used dyes, this endpoint analysis clearly demonstrates that signal-mediated nuclear import as well as export are functional in astrocytes.

To examine the ability of astrocytes to import arginine-rich NLS, we attempted to perform similar experiments with peptides containing the ARM motives of Rev or Tat conjugated with fluorescently labelled BSA. Although technical difficulties associated with coupling of the Argine rich sequences to BSA could eventually be overcome, we did not observe transport of these substrates into the nuclei of microinjected cells (data not shown). In addition, injected Rev-ARM-BSA conjugates frequently formed extra- and intracytoplasmic fluorescent granules, indicating precipitation. Intranuclear injections resulted in weak accumulation of fluorescence in the nucleoli, indicating the ability of the ARM motif to interact with nucleolar components (data not shown). Further experiments are required to assess the suitability of this type of experimental approach in examining the import activities of ARM-motifs.

Analysis of nucleocytoplasmic protein trafficking in living cells

To compare nucleocytoplasmic trafficking of shuttle proteins in various cell types we combined technology for intracellular localization of GFP-tagged proteins with an optimized cell-fusion technique. In principle, transfected cells expressing GFP-tagged proteins are cocultured with an excess of untransfected cells at high density, and fusion of adjacent cells is initiated by treatment with polyethylene glycol (PEG). Cell fusion disturbs the balance of the shuttle protein between nuclear and cytoplasmic compartments. Restoration of the steady-state distribution of the protein within the cell fusions requires the protein to shuttle (i.e. to exit the donor nucleus into the surrounding cytoplasm and enter acceptor nuclei). Sensitive time-lapse imaging under continuous culture conditions monitors migration of the GFP-tagged protein. Previously, we have successfully used this methodology to study trafficking properties of the van-Hippel-Lindau (VHL) tumor suppressor protein in HeLa cells (Lee et al., 1999).

To demonstrate that astrocytes support rapid nucleocytoplasmic protein shuttling, we assessed migration of Ran/TC4, which is believed to shuttle extremely rapidly (Gorlich and Kutay, 1999), in homo-polykaryons of U138MG astrocytic cells and HeLa control cells. In both cell types, Ran/TC4-GFP was evident in acceptor nuclei early after fusion (4 minutes) and continued accumulation was apparent for the duration of imaging (Fig. 4A). These observations indicate that PEG-treatment immediately initiates massive cytoplasmic exchange in both cell types, well before cytoplasmic boundaries appear dissolved in phase-contrast images (after approximately 30 minutes, data not shown). Furthermore, this assay demonstrates similar rapid trafficking of Ran/TC4 in both astrocytic and HeLa cells.

As an additional control for the shuttling assay, we investigated the migration behavior of B23, which displays a nuclear/nucleolar localization pattern very similar to Rev (Fig. 2A). B23 was one of the first proteins proposed to shuttle between the nucleus and cytoplasm, although at a very slow rate (16-72 hours) (Borer et al., 1989). Consequently, we did not observe migration of B23-GFP in HeLa homo-polykaryons within the 160-minute time frame of the experiment shown in Fig. 4B or in other experiments in which imaging was extended for up to 6 hours post fusion (data not shown). The only instance in which B23-GFP appeared in acceptor nuclei of polykaryons involved division of the B23-

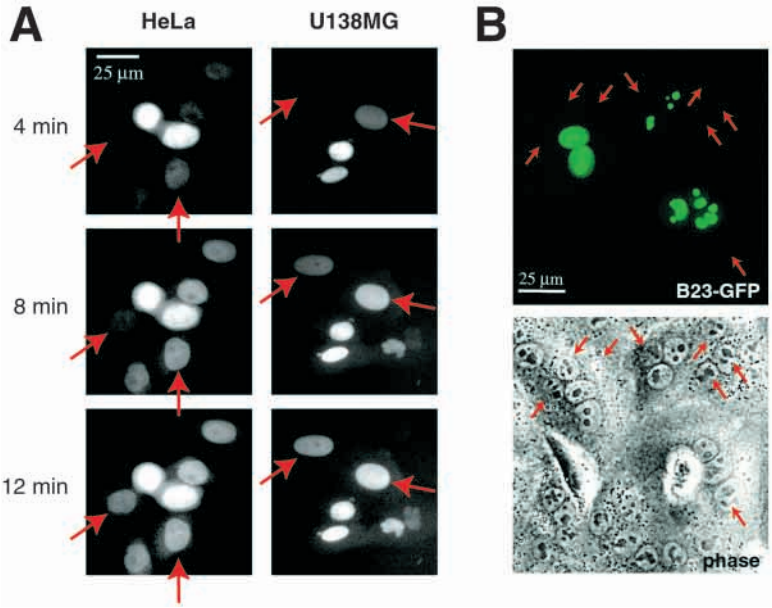

Fig. 4. Trafficking behavior of exemplary rapid and slowly shuttling GFP-tagged proteins in cell-fusion assays. (A) The panel shows the shuttling of Ran/TC4 in HeLa and U138MG homo-polykaryons. Appearance of Ran/TC4 in acceptor nuclei at 4 minutes after initiation of fusion demonstrated rapid and efficient transport of Ran/TC4 in HeLa and astrocyte homo-polykaryons. Arrows denote exemplary acceptor nuclei accumulating Ran/TC4. (B) Migration behavior of B23-GFP in HeLa cell homo-polykaryons. HeLa cells expressing B23-GFP were fused with untransfected HeLa cells. Images made at 150 minutes post-fusion show no accumulation of B23-GFP in acceptor nuclei (marked by arrows). Efficient fusion is indicated by proximity of donor and acceptor nuclei in the phase-contrast image. Images in panel B are taken from photographic slides.

GFP donor nucleus (data not shown), proving the ability of B23-GFP to efficiently enter nuclei once in the cytoplasm. This agrees with the previously shown cytoplasmic redistribution of B23 after breakdown of the nuclear envelope during mitosis (Zatsepina et al., 1997). The lack of general B23-GFP translocation indicates that the nuclear envelope remains intact for at least 6 hours post fusion in these cell-fusion assays.

Disturbed Rev-trafficking in human astrocytes

To compare nucleocytoplasmic shuttling behavior of Rev in astrocytes and Rev-permissive cells, we used 85HG66 astrocytoma cells and HeLa control cells to monitor migration of Rev-GFP in cell-fusion assays using highly sensitive time-lapse imaging, as outlined above. Cells transfected with Rev-GFP were fused with an excess of non-transfected cells lacking Rev and images taken automatically every two minutes as detailed in Materials and

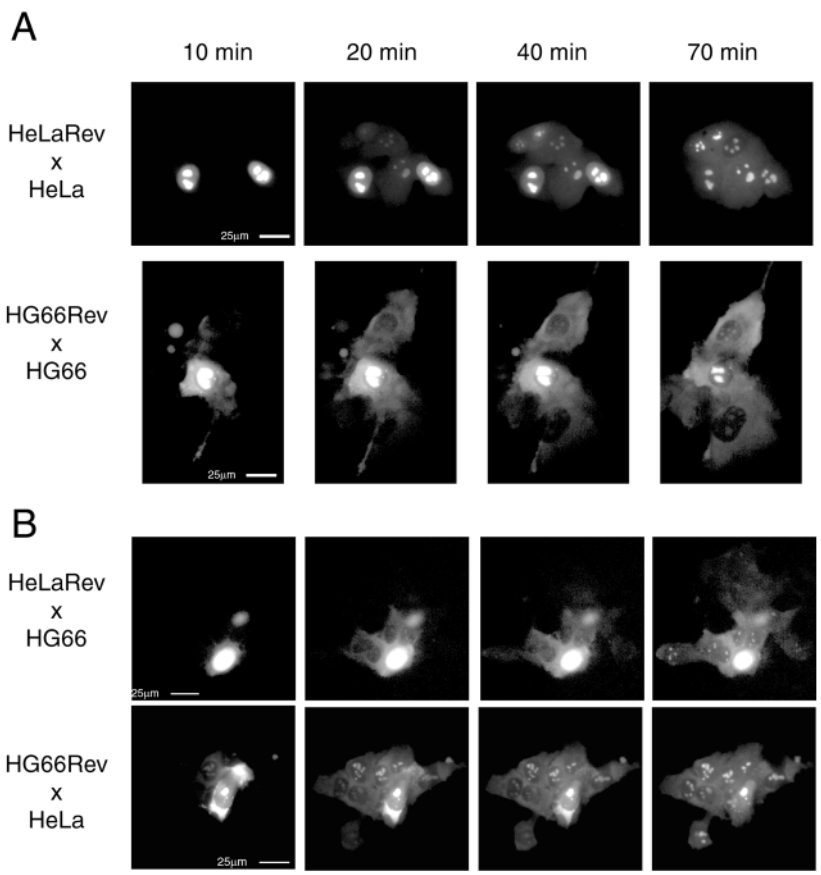

Fig. 5. Comparison of trafficking behavior of Rev-GFP in astrocytes and HeLa cells in cell-fusion assays. Trafficking properties of Rev differ in astrocytes and in HeLa cells. HeLa cells or 85HG66 (HG66) astrocytes were transfected with Rev-GFP expression plasmid pCsRevsg143 and fused with untransfected cells (for details, see text). Appropriate fields were selected and imaging begun 5-10 minutes after initiation of fusion. Four time points of representative cell-fusion experiments are shown, using identical settings for contrast and normalization of serial images. Exposure settings differed between cell-fusion experiments. (A) Homo-polykaryons. Fusions of cells of the same type. (B) Hetero-polykaryons. Heterologous cell fusions. HeLaRev×HG66: Rev-expressing HeLa cells fused with an excess of 85HG66 cells. HG66Rev×HeLa: Rev-expressing 85HG66 cells fused with an excess of HeLa cells. Accumulation of Rev-GFP in acceptor nuclei is visibly delayed in astrocyte homo-polykaryons and in hetero-polykaryons containing an excess of astrocytes. QuickTime movies of images taken every two minutes can be viewed at http://www.biologists.com/JCS/movies/jcs1709.html.

Methods. In addition, images of exemplary time points are presented in Fig. 5. In HeLa homo-polykaryons, Rev-GFP visibly accumulates in acceptor nuclei earlier than 20 minutes post fusion (Fig. 5A, top panel), indicating rapid release from donor nuclei and uptake into acceptor nuclei (e.g. shuttling) of Rev-GFP in these cells. Rev-GFP fluorescence continued to accumulate in acceptor nuclei, resulting eventually in similar brightness of fluorescent signal in all nuclei of the polykaryon one to two hours post fusion, depending on the size of the polykaryon. In homo-polykaryons of 85HG66 astrocytoma cells, Rev-GFP was released into the surrounding cytoplasm within 20 minutes post fusion, but uptake of Rev-GFP in acceptor nuclei was only barely visible 40 minutes after fusion (Fig. 5A, bottom panel). Delayed occurrence of Rev-GFP in acceptor nuclei of astrocytic homo-polykaryons was confirmed in multiple experiments with 85HG66 astrocytoma cell line. Experiments performed with U138MG astrocytoma cells yielded essentially the same results (data not shown).

Trafficking of Rev-GFP was also monitored in hetero-polykaryons generated by fusing Rev-expressing cells of one cell type with an excess of cells without Rev of the other type (Fig. 5B). To ensure that only transfected donor cells of one cell type were present in the polykaryons, GFP expressing donor cells were separated from non-transfected cells by FACS. Alternatively, donor cells were plated very thinly and microinjected with the Rev-GFP expression plasmid before seeding the non-expressing acceptor cells of the opposite type. Hetero-polykaryons generated with Rev-expressing HeLa cells as donors and an excess of 85HG66 astrocytoma acceptor cells (HeLaRev×HG66) showed similar retarded accumulation of Rev-GFP signal in donor nuclei observed in astrocyte homo-polykaryons (compare Fig. 5B, top panel, with Fig. 5A, bottom panel). In hetero-polykaryons containing Rev-expressing astrocytes as donors and an excess of HeLa acceptor cells (HG66Rev×HeLa), accumulation of Rev was more prominent in acceptor nuclei, especially in nucleoli (Fig. 5B, bottom panel). However, quantification of changes in fluorescent signal of acceptor nuclei over time indicated that nuclear accumulation of Rev-GFP in these hetero-polykaryons was still delayed compared with HeLa homo-polykaryons (see below and Fig. 6).

In printed and screen images, changes in signal intensities are often not readily apparent because the restricted gray-scale space of these display media severely limits the range of differences in signal intensity detectable by visual inspection. Therefore we took advantage of the large dynamic range of the CCD-detector to quantify changes in levels of nuclear fluorescent signal in cell-fusion experiments. Fluorescent signals in donor and acceptor nuclei of suitable polykaryons (selected as described in Materials and Methods) were quantified at various time points and relative changes in signal intensities calculated over time (Fig. 6). Measurements were performed during the period up to 30 minutes post-fusion to ensure maximum translocation capacity of fused cells. Astrocyte homo-polykaryons showed somewhat slower rates of decrease of fluorescent signal from donor nuclei than HeLa homo-polykaryons (Fig. 6A, compare HG66Rev×HG66 and HeLaRev×HeLa plots). In hetero-polykaryons (HG66Rev×HeLa and HeLaRev×HG66), fluorescent signal of donor nuclei decreased at a rate comparable with HeLa cell homo-polykaryons.

Rates of increase of fluorescent signal in acceptor nuclei differed dramatically in both cell types (Fig. 6B). HeLa homo-polykaryons showed much more rapid increase of fluorescent signal than astrocyte homo-polykaryons. Fusion of HeLa cells with 85HG66 astrocytes strongly reduced the accumulation rates of Rev-GFP in acceptor nuclei of hetero-polykaryons to levels comparable with astrocyte homo-polykaryons (HG66Rev×HG66). This was observed both when Rev-expressing astrocytes were fused with an excess of HeLa cells (HG66Rev×HeLa) and in reverse fusions (HeLaRev×HG66).

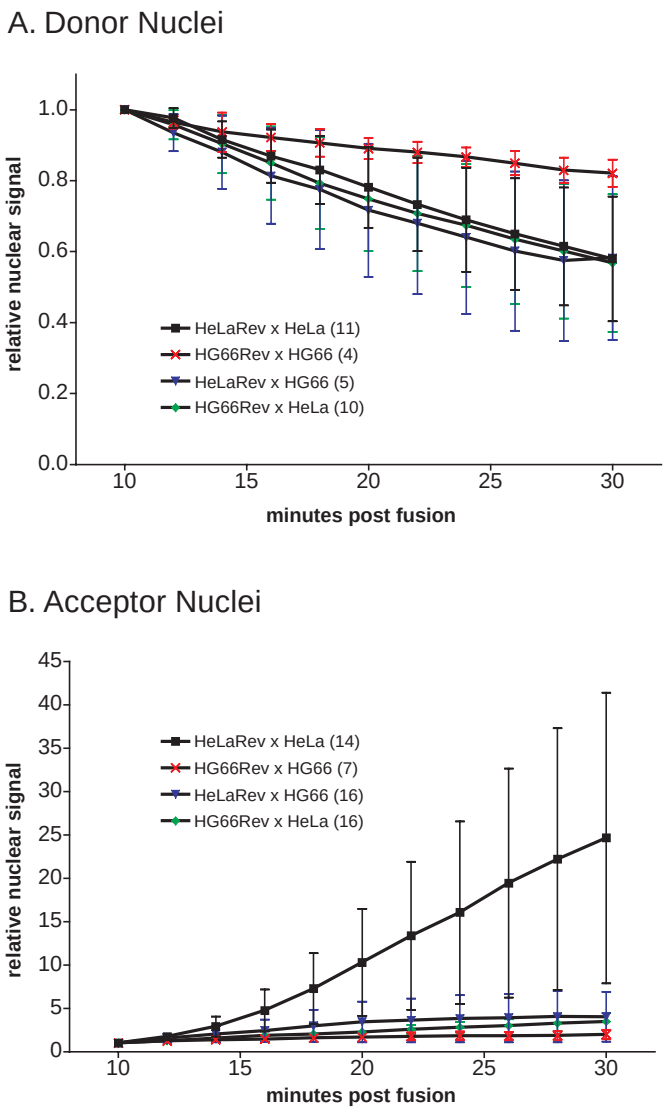

Fig. 6. Quantitative analysis of temporal changes of nuclear signal for Rev-GFP in cell-fusion assays. (A) Decrease of Rev-GFP fluorescence in donor nuclei. (B) Increase of Rev-GFP fluorescence in acceptor nuclei. Cell-fusion experiments performed with HeLa (HeLa) and astrocytoma 85HG66 (HG66) cell lines were selected for quantitative analysis, as detailed in Materials and Methods. Combinations of fused cells are indicated in the key for each figure, with the cell type transfected with Rev-GFP expression plasmid given first and the untransfected cell type provided in excess given second. The fluorescent signal of each nucleus was quantified in serial images taken every two minutes and related to the signal of the same nucleus in the first image (set at 1). All nuclear signals were corrected for background fluorescence. The total number of nuclei analyzed for each cell combination is indicated in brackets. Data points represent relative mean fluorescence intensities plotted versus time. Error bars indicate 95% confidence intervals.

Thus reduced nuclear accumulation of Rev in hetero-polykaryons does not appear to depend on the cellular source of Rev-GFP or on the nuclei involved in trafficking. Rather, these results indicate that delayed nuclear accumulation of Rev-GFP in hetero-polykaryons is mediated by the cytoplasm of astrocytes.

From these cell-fusion assays we conclude that Rev shuttles in astrocytes, but does so with different dynamics than in Rev-permissive cells. In particular, nuclear accumulation of Rev is dramatically reduced in astrocytes. Heterologous fusions between astrocytes and Rev-permissive cells (HeLa) indicate that the activity inhibiting nuclear accumulation of Rev is contained in the cytoplasm of astrocytes and can be transmitted to Rev-permissive cells.

DISCUSSION

Disruption of steady-state distribution of Rev in astrocytes

The shortage of information concerning nucleocytoplasmic transport processes in human brain cells, particularly astrocytes, led us to investigate general features of nuclear transport in these cells. Here, we show that the major transport receptors importin β and exportin 1 are expressed in astrocytes and display similar intracellular localization patterns as in HeLa cells. In addition, various proteins known to interact with these transport receptors showed similar localization characteristics in astrocytes and HeLa cells. Nuclear localization was observed for proteins with various signals recognized by importin β, including the export-deficient Rev mutant M10BL and conjugates between bovine serum albumin (BSA) and the 'classical' lysine-rich NLS of SV-40 large T-antigen. Cytoplasmic accumulation was evident for proteins with exportin-1-dependent nuclear export signals, including PKI and conjugates between Rev NES and BSA. Furthermore, astrocytes showed expected nuclear localization and rapid and efficient trafficking of the small GTPase Ran-TC4, a shuttle protein that is crucial for nucleocytoplasmic transport (Kehlenbach et al., 1998; Lounsbury et al., 1996; Smith et al., 1998). These results indicate that there are no global differences in the nucleocytoplasmic transport between astrocytes and HeLa cells. However, steady state localization of the HIV-1 Rev shuttle protein is different in astrocytes from Rev-permissive control cells, with high levels of Rev contained in the astrocytic cytoplasm (Fig. 1). In addition, preliminary studies indicate that Rev from another lentivirus, the simian immunodeficiency virus (SIV), is redistributed to the cytoplasm of human astrocytes (M. Neumann, unpublished). Cytoplasmic accumulation of HIV-1 Rev has been associated with inactivation of Rev in many instances (Hope et al., 1990; Ludwig et al., 1999; Malim et al., 1989; Neumann et al., 1995; Stauber et al., 1998a; Szilvay et al., 1997), demonstrating the importance of nuclear accumulation for the functionality of Rev.

HIV-Tat, like Rev, has an ARM motif, which serves as an NLS (Truant and Cullen, 1999). We did not observe an astrocyte-specific difference in localization behavior of Tat-GFP. Notably, Tat showed significantly more cytoplasmic accumulation in HeLa cells than Rev, which is interesting because no export signal for Tat has been identified. From these studies, we conclude that accumulation of Rev in the cytoplasm of astrocytes does not involve solely the ARM of Rev, which is similar to that of Tat. Rather, sequences outside the ARM may play an important role in altered localization and shuttling behavior of Rev in astrocytes.

We conclude that astrocytes have biological properties that specifically perturb crucial nuclear accumulation of lentiviral Rev proteins.

Astrocytes show diminished nuclear uptake of Rev during shuttling

We assessed shuttling of Rev in different cellular backgrounds by monitoring nuclear translocation of endogenously expressed Rev-GFP in living cells by sensitive time-lapse image analysis. This methodology has two advantages over other assays used to address nuclear transport. First, the protein under study is produced intracellularly, in contrast to microinjection (Bevec et al., 1996) or cell-permeabilization assays (Gorlich et al., 1995) in which bacterially produced recombinant proteins are added to cells. Second, the fluorescent tag allows continuous monitoring of protein transport in the same cellular background, whereas in other hetero-polykaryon assays proteins are detected with antibodies in different cells fixed and permeabilized at the same time point (Borer et al., 1989; Schmidt-Zachmann et al., 1993). In our assay, rapid and efficient cytoplasmic exchange was confirmed by rapid appearance of the Ran-TC4 shuttle protein in acceptor nuclei of polykaryons. In addition, accumulation of Ran-GFP in acceptor nuclei and sustained retention of B23 in donor nuclei support structural integrity of nuclei in polykaryons. Failure to observe B23-GFP trafficking in intact nuclei of polykaryons within a 6-hour time frame is presumably due to the fact that, compared with Rev (this study), B23 shuttles very slowly (16-72 hours; Borer et al., 1989). Notably, the ability of B23-GFP to enter and accumulate in acceptor nuclei of cell fusions was confirmed in one experiment in which the donor nucleus underwent division (data not shown). This raises the possibility that the shuttling of B23 is most relevant for non- or slowly dividing cells.

Using this cell-fusion assay, we demonstrated that Rev traffics with different dynamics in astrocytes and HeLa cells (Fig. 5; Fig. 6; and see movies at http://www.biologists.com/JCS/movies/jcs1709.html). The decrease in Rev-GFP signal in donor nuclei was somewhat slower in astrocytes than in HeLa cells, indicating that cytoplasmic accumulation of Rev is not caused by accelerated expulsion of Rev from nuclei. By contrast, astrocytes showed dramatically decreased rates of accumulation of Rev in acceptor nuclei, compared with rates in HeLa cells. This does not appear to be caused by an inherent failure of astrocytic nuclei to retain Rev after entry, because the export deficient Rev mutant M10BL accumulates to high levels in astrocytic nuclei (Fig. 1B). Rather, our results indicate diminished nuclear uptake of Rev in astrocytes. This conclusion is further supported by a separate study demonstrating slower rates of nuclear accumulation of Rev in astrocytes than in HeLa cells in the presence of the nuclear export inhibitor Leptomycin B (E. Afonina et al., unpublished). Heterologous cell fusions between astrocytes and Rev-permissive cells also showed diminished nuclear accumulation of Rev. These results are consistent with the presence of an inhibitory activity in the cytoplasm of astrocytes that prevents efficient nuclear uptake of Rev during shuttling, by retaining Rev in the cytoplasm. The observation that the cytoplasmic inhibitory activity of astrocytes can be transmitted to other cells suggests exploration of its therapeutic potential in future studies.

Possible mechanisms for retarded nuclear uptake of Rev in astrocytes

Several mechanisms involved in inhibiting nuclear import of other proteins may be relevant to limiting nuclear uptake of Rev in astrocytes. Cytoplasmic accumulation and retarded nuclear uptake of Rev could involve masking of nuclear import signals by association with cytoplasmic factors and/or by induction of conformational changes of Rev by the astrocytic cytoplasm. Masking of nuclear localization signals is a frequently observed mechanism for inactivation of inducible nuclear transcription factors, such as NF-κB and NF-AT (nuclear factor of activated T cells) by sequestration in the cytoplasm. For example, nuclear translocation of NF-κB is prevented by interaction with IκB, which binds to and masks the NLS on NF-κB (Beg et al., 1992; Henkel et al., 1992). Cytoplasmic accumulation of NF-AT involves intramolecular masking by an NLS-masking domain within NF-AT (Zhu et al., 1998). In these examples, masking and unmasking of NLS is regulated by phosphorylation steps in rapid response to external signals. Several studies have demonstrated phosphorylation of Rev by protein kinases known to be involved in signal transduction pathways (Cochrane et al., 1989; Hauber et al., 1988; Meggio et al., 1996; Ohtsuki et al., 1998; Yang and Gabuzda, 1999). Phosphorylation of Rev has been shown to induce a conformational change in Rev protein structure in vitro (Fouts et al., 1997), suggesting that it plays a role in regulating biological properties of Rev. Preliminary studies show that phosphorylation of Rev occurs in astrocytes at overall levels similar to HeLa cells (D. D'Agostino, and M. Neumann, unpublished). The role of Rev phosphorylation in alterations of its trafficking properties in astrocytes should be addressed in more detail in future studies.

Another possibility for retarded nuclear import of Rev in astrocytes is abnormal exposure of signals within Rev that promote cytoplasmic retention and/or inhibit nuclear uptake. Various proteins have been shown to contain cytoplasmic retention signals in addition to nuclear localization signals, including the transcription factor ERK2 (Rubinfeld et al., 1999; Zhu et al., 1998), DNA methyltransferase (Cardoso and Leonhardt, 1999), LKB1 serine/threonine kinase (Nezu et al., 1999) and Cyclin B (Yoshitome et al., 1998). Rev has been proposed to contain a signal in the N-terminal region inhibiting nuclear entry (Kubota and Pomerantz, 1998). Changes in the conformation of Rev in astrocytes could promote the activity of this or other signals that inhibit nuclear entry of Rev. However, the same signal was later suggested to counteract nuclear diffusion of Rev (Kubota and Pomerantz, 2000), indicating that the role of this sequence in nuclear transport of Rev is still unclear.

Finally, cytoplasmic accumulation of nuclear proteins can be mediated by tethering to cytoskeletal components. This is exemplified by interaction of the influenza virus nucleoprotein (NP) with F-actin for cytoplasmic retention of ribonucleoproteins during virus assembly (Digard et al., 1999). However, the diffuse localization pattern of Rev in the cytoplasm of astrocytes argues against anchorage of Rev to cytoskeletal structures in astrocytes, although this needs to be studied in more detail.

Importance of nucleocytoplasmic trafficking for virus replication

The overall picture derived from studying biological properties of Rev in astrocytes suggests a model in which Rev is prevented from functioning efficiently in astrocytes by retention of Rev in the cytoplasm, causing diminished nuclear uptake of Rev during shuttling. Next to restrictions at the level of HIV entry, defective Rev-function is an important mechanism in limiting HIV-replication in astrocytes (reviewed by Brack-Werner and Bell, 1999). This study provides evidence that trafficking properties of Rev can differ between various cell types and may therefore influence overall rates of virus production by infected cells. Further studies are required to determine whether altered trafficking dynamics of Rev are limited to astrocytes or are observed in other HIV reservoir cells, such as resting T cells (Finzi et al., 1999) and dendritic cells (Canque et al., 1999).

On a more general note, many viruses depend on cellular nucleocytoplasmic transport pathways for the replication of their genomes, temporal regulation of viral gene expression and assembly of viral components (reviewed by Cullen, 1998b; Kasamatsu and Nakanishi, 1998; Whittaker et al., 1996). Even viruses that are thought to replicate exclusively in the cytoplasm, such as members of the Flaviviridae and of the Togaviridae, have been shown to encode proteins that use nucleocytoplasmic transport pathways (Forwood et al., 1999; Kim et al., 1999; Rikkonen, 1996). In addition, several viruses encode regulatory factors which, like Rev, shuttle between nucleus and cytoplasm and mediate the selective export of viral RNAs, thus stimulating viral gene expression on post-transcriptional level. Examples include the Rex protein of the human T-cell leukemia viruses (Hakata et al., 1998; Kusuhara et al., 1999), the immediate-early protein ICP27 of the herpes simplex virus (Mears and Rice, 1998; Sandri-Goldin, 1998; Soliman et al., 1997) and its functional homologue in Herpesvirus 8 (Bello et al., 1999), and a complex of the Adenovirus early proteins E1B 55 kDa and E4 34 kDa (Dobbelstein et al., 1997). Our studies of Rev transport in astrocytes suggest that cells specifically modulate nucleocytoplasmic trafficking properties of crucial viral regulatory factors and thus control virus replication. Identification of cellular factors that specifically perturb nucleocytoplasmic transport of crucial viral proteins may be valuable antiviral weapons in combating the replication of cytopathic viruses in infected cells.

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