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Vpx proteins of SIVmac239 and HIV-2ROD interact with the cytoskeletal protein α-actinin 1

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vpx genes of human immunodeficiency virus type 2 (HIV-2) and immunodeficiency viruses from macaques (SIVmac), sooty mangabeys (SIVsm) and red-capped mangabeys (SIVrcm) encode a 112 aa protein that is packed into virion particles via interaction with the p6 domain of $p55^{gag}$. Vpx localizes to the nucleus when expressed in the absence of other viral proteins. Moreover, Vpx is necessary for efficient nuclear import of the pre-integration complex (PIC) and critical for virus replication in quiescent cells, such as terminally differentiated macrophages and memory T cells. Vpx does not contain sequence elements that are homologous to previously characterized nuclear localization signals (NLSs). Therefore, it is likely that Vpx-dependent import of the PIC is mediated by interaction of Vpx with cellular proteins that do not belong to the classical import pathways. By using a yeast two-hybrid screen, α-actinin 1, a cytoskeletal protein, was identified to interact with SIVmac239 Vpx. Interestingly, deletion of the proline-rich C-terminal domain (aa 101–112) of Vpx, which is important for nuclear localization, resulted in loss of interaction with α-actinin 1. These findings suggest that the interaction with α-actinin 1 may play an important role in the transport of Vpx to the nucleus and in Vpx-mediated nuclear import of the PIC.

INTRODUCTION

One of the properties of lentiviruses is their genetic complexity. Human immunodeficiency virus types 1 and 2 (HIV-1 and -2) and the various simian immunodeficiency viruses (SIVs), which naturally infect more than 20 non-human primate species (Hahn *et al.*, 2000), encode several accessory and/or regulatory genes in addition to the structural *gag*, *pol* and *env* genes that are present in all retroviruses (Cullen, 1998; Trono, 1998). Human immunodeficiency viruses and the different SIVs share significant genetic homology. HIV-2 and the closely related simian immunodeficiency viruses from macaques, sooty mangabeys and red-capped mangabeys (Beer *et al.*, 2001) contain a *vpx* gene, as well as the evolutionarily related *vpr* gene. Due to the sequence similarity of Vpx and Vpr, it has been discussed that the Vpx protein arose from a gene-duplication event (Tristem *et al.*, 1990, 1992). However, *vpx* is absent from HIV-1 and most other known SIVs (Takemura & Hayami, 2004). It has been shown that SIVsm Vpr and Vpx proteins have distinct, non-complementary functions (Fletcher *et al.*, 1996). Whilst Vpr induces cell-cycle arrest at the G2/M stage of the cell cycle (Fletcher *et al.*, 1996; Mueller & Lang, 2002; Stivahtis *et al.*, 1997; Zhu *et al.*, 2001), Vpx is involved in nuclear import of the viral pre-integration complex (PIC) (Depienne *et al.*, 2000; Fletcher *et al.*, 1996; Pancio *et al.*, 2000). Animal studies showed that both proteins contribute to viral pathogenicity, as rhesus monkeys infected with SIVmacΔ*vpx* virus or with Δ*vpr*Δ*vpx* double-mutant SIV had lower virus loads than animals infected with wild-type virus and progression to AIDS was delayed or absent (Gibbs *et al.*, 1995). Additionally, Hirsch *et al.* (1998) showed that Vpx is essential for efficient dissemination and spread of SIVsm following mucosal and intravenous infection of macaques. Recent data indicate that Vpx is critical for upregulation of HIV-2 replication in natural target cells by enhancing nuclear import of the viral genome (Ueno *et al.*, 2003). In conclusion, these results indicate that Vpx is important for virus load and disease progression in lentiviruses of the HIV-2/SIVmac lineage.

Vpx is a 12 kDa, 112 aa protein that is highly conserved between SIVmac and HIV-2 (Henderson *et al.*, 1988; Kappes *et al.*, 1988; Yu *et al.*, 1988) (Fig. 1). Interaction of Vpx with the C-terminal proline-rich portion of the Gag precursor allows packaging of an amount comparable to that of Gag

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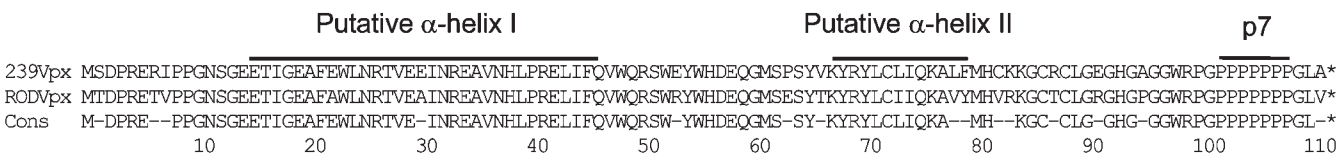


Fig. 1. Alignment of the amino acid sequences of Vpx proteins from SIVmac239 (239Vpx) and HIV-2ROD (RODVpx). Sequences were obtained from the Los Alamos database. The positions of the two predicted amphipathic α-helical structures extending from aa 18 to 49 and 71 to 82 and the C-terminal proline-rich domain (p7) extending from aa 103 to 109 are indicated (Mahalingam *et al.*, 2001). The consensus sequence (cons) is indicated. Identical amino acids are shown in capital letters; a dash (–) indicates a difference between SIVmac239 and HIV-2ROD in the consensus sequence.

proteins into virus particles (Henderson *et al.*, 1988; Pancio & Ratner, 1998; Selig *et al.*, 1999; Wu *et al.*, 1994). The presence of Vpx in the virion suggests an important function in the early life cycle of the virus. One function that has already been demonstrated for SIVsm Vpx and HIV-2 Vpx is to direct the nuclear import of the PIC in quiescent cells (Hirsch *et al.*, 1998; Pancio *et al.*, 2000), a prerequisite for the integration of viral DNA into the host genome (Bukrinsky *et al.*, 1993a; Emerman, 1996; Gallay *et al.*, 1997). The observation that SIVmac/SIVsm lacking Vpx has a significantly reduced ability to replicate in terminally differentiated macaque macrophages (Fletcher *et al.*, 1996; Gibbs *et al.*, 1994) and memory T cells (Hirsch *et al.*, 1998) presumably results from this deficit in PIC transport.

Despite the fact that Vpx is required for HIV-2/SIVsm/SIVmac dissemination *in vivo* and replication in non-dividing cells, only a few conclusive analyses combining structural and functional data concerning the Vpx protein have been reported. Furthermore, the mechanism by which Vpx mediates nuclear import of the HIV-2/SIVsm/SIVmac PIC remains unknown. Immunofluorescence studies have shown that Vpx localizes to the plasma membrane in cells infected with HIV-2/SIVsm (Kappes *et al.*, 1993) but that, when expressed in the absence of other viral proteins, it localizes to the cytoplasm, as well as to the nucleus (Di Marzio *et al.*, 1995; Kappes *et al.*, 1993; Pancio *et al.*, 2000).

Moreover, Pancio *et al.* (2000) reported that deletion of the proline-rich C terminus (aa 102–112) of the HIV-2ROD Vpx protein abrogates nuclear localization and attenuates HIV-2 replication in macrophages. These data suggest that deletion of the C-terminal proline-rich domain of Vpx is linked to functional loss of Vpx-mediated PIC transport to the nucleus. Other studies showed that the proline-rich C terminus of Vpx is not sufficient for nuclear localization (Belshan & Ratner, 2003; Mahalingam *et al.*, 2001). As Vpx does not contain sequence elements that are homologous to previously characterized nuclear localization signals (NLSs), it may contain a novel NLS domain. Recently, Belshan & Ratner (2003) described aa 65–72 of Vpx as the minimal transferable region of Vpx that conferred nuclear import. Similar data were obtained by Mahalingam and coworkers, who identified a conserved domain within the C-terminal part of Vpx that is sufficient to mediate the transport of heterologous proteins, such as GFP and β-galactosidase (β-Gal), into the nucleus (Kumar *et al.*, 2003; Mahalingam *et al.*, 2001). Alternatively, Vpx may gain access to the nucleus by interacting with another NLS-containing protein and thus exploiting cellular nuclear import pathways. Similar interactions have been described for a number of other viral proteins (La Boissière *et al.*, 1999; Weil *et al.*, 1999). Numerous studies have shown that the cytoskeleton plays an important role in intracellular transport processes (Bearer & Satpute-Krishnan, 2002; Cudmore *et al.*, 1995; McDonald *et al.*, 2002; Sodeik, 2000; Sodeik *et al.*, 1997; Suomalainen *et al.*, 1999). In the present study, we report that SIVmac239 Vpx and HIV-2ROD Vpx interact with α-actinin 1 (Millake *et al.*, 1989), a cytoskeletal protein that belongs to the spectrin gene superfamily (Dixson *et al.*, 2003). α-Actinin 1 is an F-actin bundling protein and participates in the organization of the cytoskeleton (Kuhlman *et al.*, 1994; Wachsstock *et al.*, 1987). Several studies have provided evidence that the cytoskeleton may be involved in the assembly and budding of retroviruses. For example, it has been shown that HIV-1 Gag can bind F-actin (Liu *et al.*, 1999; Rey *et al.*, 1996); this specific cytoskeletal protein has been found in virions (Ott *et al.*, 1996).

Our results indicate that the C terminus of Vpx, which mediates the nuclear localization of HIV-2 Vpx (Pancio *et al.*, 2000), is essential for the interaction of SIVmac239 Vpx with α-actinin 1. As the interaction with α-actinin 1 is conserved between SIVmac239 Vpx and HIV-2ROD Vpx, it is likely that this interaction may play an important role in the transport of Vpx, and thus the transport of the PIC, into the nucleus.

METHODS

Plasmids. For yeast two-hybrid screening, the *vpx* ORFs of SIVmac239 and HIV-2ROD were amplified by PCR using the primer pair Y2H-239vpx-5′ (5′-CGGAATTCTCAGATCCCAGGGAGAGAATTC-3′) and Y2H-239vpx-3′ (5′-CGGATCCTTATGCTAGTCCTGGAGGGGG-3′) for SIVmac239 *vpx* and the primer pair Y2H-RODvpx-5′ (5′-CGGAATTCACAGACCCCAGAGAGACAGTA-3′) and Y2H-RODvpx-3′ (5′-CGGGATCCTTAGACCAGACCTGGAGGGG-3′) for HIV-2ROD *vpx*, respectively. Unique restriction sites for *Eco*RI and *Bam*HI (underlined) were introduced by the PCR primers. PCR products were cloned into the yeast expression vector pGBT9 (Clontech) and the correct sequence of the wild-type *vpx*

genes was verified by sequence analysis. A cDNA library from Jurkat T cells fused to the Gal4 DNA-activation domain in the vector pACT2 was purchased from Clontech. Genes that were identified in the yeast two-hybrid screens were amplified by using the primers pACT2-5 (5′-CCGCCATGGCTTACCCATACGATGTTCCAGATTACGC-3′) and AM3-AD (5′-GTGAACTTGCGGGGTTTTTCAGTATCTACGAT-3′). By using these primers, an N-terminal haemagglutinin (HA)-tagged fusion construct was obtained. The PCR products were digested either with *Xho*I and *Eco*RV or with *Eco*RV only, cloned into the expression vector pcDNA3 (Invitrogen) and sequence analyses were performed. For transient-expression studies, the *vpx* ORF of SIVmac239 was amplified by PCR using the primers 239vpx-5′ (5′-CGGGATCCTCAGATCCCAGGGAGAGAA-3′) and 239vpx-3′ (5′-CGGAATTCATGCTAGTCCTGGAGGGGG-3′). The *vpx* gene of HIV-2ROD was amplified by using the primers RODvpx-5′ (5′-CGGGATCCACAGACCCCAGAGAGACAGTA-3′) and RODvpx-3′ (5′-CGGAATTCTTAGACCAGACCTGGAGGGG-3′). Subsequently, the PCR products were cloned into the expression vector pCMV6Myc (Sells & Chernoff, 1995) by using the restriction sites for *Bam*HI and *Eco*RI (underlined) that were introduced by the PCR primers and the correct sequence of the wild-type *vpx* gene was verified. The plasmids obtained, pCMV6M 239vpx and pCMV6M RODvpx, resulted in the expression of N-terminally Myc epitope-tagged (EQKLISEEDL) Vpx proteins lacking the first methionine residue of wild-type Vpx.

Yeast two-hybrid screen and interaction analyses. Yeast two-hybrid screening was performed according to the protocol suggested by the Matchmaker two-hybrid system (Clontech). The yeast strain *Saccharomyces cerevisiae* CG-1945 was transformed sequentially with either the SIVmac239vpx or HIV-2RODvpx hybrid expression plasmids and the Jurkat cDNA library. Transformants were plated onto synthetic complete medium without tryptophan (Trp), leucine (Leu) and histidine (His) in the presence of 15 mM 3-amino-1,2,4-triazole.

His^+ colonies were tested for β-Gal activity by filter-lift assays according to the manufacturer's instructions (Clontech). Nucleic acids were extracted from $lacZ^+$ yeast colonies and transformed into *Escherichia coli* strain KC8. Plasmids from the segregates (Leu^+ Amp^+) containing only the pACT2 Jurkat cDNA plasmids were isolated and sequenced. For quantitative β-Gal assays, bait and target plasmids were transformed into *S. cerevisiae* strain Y190. Liquid β-Gal assays were performed by using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) according to the manufacturer's instructions (Clontech) with slight modifications: cells were lysed by adding SDS and chloroform to final concentrations of 0·006 % (w/v) and 0·06 % (v/v) respectively, instead of lysing by freeze–thaw cycles.

Tissue culture and transfection. COS7 cells were grown in Dulbecco's modified Eagle medium supplemented with 10 % fetal calf serum (FCS), glutamine (0·35 g l^{-1}), streptomycin (0·12 g l^{-1}) and penicillin (0·122 g l^{-1}). Transfection of cells used for transient-expression experiments was carried out according to a diethylaminoethyl Dextran protocol (Aruffo & Seed, 1987). Cells were harvested 48 h after transfection, washed once with PBS and used immediately or frozen for later use. COS7 cells used for immunofluorescence studies were transfected with Lipofectamine reagent according to the protocol suggested by GibcoBRL. Cells were harvested 48 h after transfection and were stained immediately. The hybridoma cell line Myc 1-9E10.2 (ATCC) was propagated in RPMI 1640 medium supplemented with 10 % FCS, glutamine, streptomycin and penicillin. For antibody production, cells were transferred to medium supplemented with 5 % FCS and cultivated for 5 days. The supernatant was cleared from cells and debris by centrifugation and buffered with 20 mM Tris, pH 8·0.

Immunoprecipitation and Western blot analysis. Transfected COS7 cells were lysed in NP-40 buffer (0·5 % Nonidet NP-40, 0·15 M NaCl and 50 mM HEPES, pH 7·5) that contained phosphatase and protease inhibitors (1 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, 5 μg leupeptin ml^{-1} and 28 μg aprotinin ml^{-1}). Insoluble components were removed from lysates by centrifugation at 18 000 ***g*** at 4 °C for 30 min. Immunoprecipitations were performed with either 1–2 μg anti-Vpx or anti-HA antibody or a suitable amount of Myc 9E10 hybridoma supernatant. Immune complexes were recovered by adsorption to protein A–Sepharose (Amersham Biosciences) and were washed three times with lysis buffer and once with 10 mM Tris/HCl, pH 7·5. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to an Immobilon-P membrane filter (Millipore) by using a Hoefer semi-dry unit. Membrane filters were blocked for 1 h either with PBS, 0·4 % Tween 20, 5 % non-fat dry milk or with PBS, 0·4 % Tween 20, 5 % FCS. Antibodies were diluted according to the manufacturer's recommendations. Anti-HA antibodies were pre-adsorbed onto COS7 cells for at least 1 h prior to use. Filters were incubated with the appropriate primary antibody for 1 h at room temperature or at 4 °C overnight. Subsequently, filters were washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 30–45 min at room temperature. Proteins were detected by enhanced chemiluminescence (Amersham Biosciences).

Indirect immunofluorescence. Transfected cells were fixed with 3 % paraformaldehyde in PBS for 30 min at room temperature and subsequently treated with PBS containing 0·1 % Triton X-100 to permeabilize cell membranes. Cells were washed with PBS and blocked with PBS containing 1 % BSA for 30 min. Antibodies were diluted in PBS according to the manufacturer's recommendations. Cells were incubated with the appropriate antibody for 30 min at room temperature. Nuclei were stained with DAPI (3 μg ml^{-1}; Roche) before embedding. Immunofluorescence images were analysed with an Axioplan 2 microscope (Zeiss) or with a confocal microscope (Leica).

Antibodies and antisera. Anti-Vpx antisera were generated by immunization of rabbits with purified glutathione *S*-transferase (GST)–SIVmac239 Vpx fusion protein. Obtained antibodies showed comparable reactivity against SIVmac239 Vpx and HIV-2ROD Vpx proteins. An anti-α-actinin mAb (clone BM-75.2) was purchased from Sigma. HA epitope-specific antibodies were obtained from BAbCo and Myc epitope-specific antibodies were produced by using the hybridoma cell line Myc 1-9E10.2. HRP-conjugated secondary antibodies were purchased from Santa Cruz or DAKO. Anti-Vpx antibodies were used at a 1 : 1000 dilution, whereas anti-Myc supernatant was used at a 1 : 50 dilution. For immunofluorescence studies, anti-Vpx polyclonal antibodies and Alexa Fluor 488-conjugated anti-HA mAbs (Molecular Probes), in combination with Texas red-conjugated anti-rabbit antibodies (Molecular Probes), were used in order to visualize the proteins by confocal microscopy.

Construction of SIVmac239 *vpx* deletion variants. In order to map the protein-interaction domains within SIVmac239 Vpx, several N- and C-terminal deletion mutants (designated ΔN1, ΔN2, ΔN3, ΔC1, ΔC2 and ΔC3) were created. Plasmid pCMV6M 239vpx, containing the wild-type ORF of SIVmac239 *vpx*, was used as the template for PCR amplification of the truncated *vpx* fragments. Fragments ΔN1, ΔN2 and ΔN3 were amplified by using the 5′ primers ΔN1 (5′-CGGGATCCAGTGGAGAAGAGACAATAGGAG-3′, positions 6004–6025), ΔN2 (5′-CGGGATCCAACAGAACAGTAGAGGAGATA-3′, positions 6143–6163), ΔN3 (5′-CGGGATCCGCGGTAAACCACCTACCAAG-3′, positions 6173–6192) and the 3′ primer 239vpx-3′. Fragments ΔC1, ΔC2 and ΔC3 were amplified by using the 5′ primer 239vpx-5′ and the 3′ primers ΔC1 (5′-CGGAATTCTTATGGTCTCCATCCCCCTGC-3′, positions 6370–6353), ΔC2 (5′-CGGAATTCTTAACAGCCTTTCTTGCAATGCAT-3′, positions 6328–6307) or ΔC3 (5′-CGGAATTCTATATTAAACACAAGTATCTGTATTT-3′, positions 6292–6268). Unique restriction sites

for *Bam*HI and *Eco*RI (underlined) were introduced by the PCR primers. PCR products were cloned into the prokaryotic expression vector pGex2TK (Amersham Biosciences) and the correct sequence of the mutant *vpx* genes was verified. Obtained plasmids resulted in the expression of N-terminally GST-tagged Vpx proteins.

Expression and purification of recombinant GST fusion proteins. *E. coli* XL2 Blue cells (Stratagene) were transformed with the bacterial expression vector pGEX-2TK, pGEX-2TK 239vpx or plasmids encoding the *vpx* deletion variants. GST–Vpx fusion proteins were expressed in *E. coli* XL2 Blue cells following induction by using IPTG (1 mM final concentration) and purified by binding to glutathione–Sepharose beads (Amersham Biosciences) as described by Smith & Johnson (1988).

***In vitro* binding assay.** Cells expressing the cytoskeletal protein α-actinin 1 were lysed in NP-40 buffer containing phosphatase and protease inhibitors. Cleared cell lysates were mixed with 2–5 μg GST–Vpx fusion proteins or GST alone, bound to 25–50 μl glutathione–Sepharose beads and incubated for 2–4 h at 4 °C. The affinity-purified proteins were washed with NP-40 buffer. For expression controls, GST–Vpx wild-type and mutant proteins were separated by SDS-PAGE and stained with Coomassie blue.

DNA sequencing. Sequence analyses were performed with a Big Dye Terminator cycle sequencing ready reaction kit from Perkin Elmer. The procedure was carried out according to the manufacturer's recommendations. All analyses were carried out on an ABI Prism 377 DNA sequencer (Applied Biosystems).

Bioinformatics. DNA sequence analysis, including multiple alignments and predicted translations, was performed with the Wisconsin Package version 10.1 from GCG (Genetics Computer Group) (Devereux *et al.*, 1984). BLAST searches were carried out with the NCBI MEGABLAST system.

RESULTS

Identification of α-actinin 1 as a cellular interaction partner of SIVmac239 and HIV-2ROD Vpx by yeast two-hybrid experiments

To identify possible cellular interaction partners of SIVmac239 Vpx and HIV-2ROD Vpx, two independent yeast two-hybrid screens were carried out. Expression of Gal4–Vpx fusion proteins of SIVmac239 and HIV-2ROD in yeast (*S. cerevisiae* CG-1945^e) was confirmed by Western blot analysis (data not shown). In order to exclude the possibility that bait proteins are able to activate transcription in yeast by themselves, β-Gal expression of the yeast cells (CG-1945^e) transformed with the Gal4–Vpx constructs was tested by filter-lift experiments. No β-Gal expression could be detected with this combination. A Jurkat cDNA library fused to the Gal4 activation domain was introduced into yeast cells that had previously been transformed with Gal4–Vpx expression plasmids. Transformants harbouring interacting proteins were selected in the absence of histidine. For 239Vpx, $3{\cdot}0 \times 10^5$ transformants were plated onto selective synthetic medium, whilst for RODVpx, $4{\cdot}3 \times 10^6$ transformants were plated. His$^+$ transformants were screened for their ability to produce β-Gal by using a filter-lift assay (data not shown). Out of 256 *HIS3*-positive clones that were identified in the SIVmac239 Vpx screen, 36 tested positive for β-Gal activity. In the HIV-2ROD Vpx screen, 53 His$^+$ clones were identified; 45 of these were able to produce β-Gal (data not shown). Sequence analysis and a search for homologies in the NCBI database revealed that two clones that were identified in the 239Vpx screen showed 99 % nucleotide sequence identity with the human α-actinin 1 protein, encoded by the ACTN1 gene. Comparison of the amino acid sequence encoded by these two clones showed that one clone encoded aa 1–892 of α-actinin 1 (full-length α-actinin 1) and the other encoded aa 316–892 of α-actinin 1. From the 45 clones isolated in the HIV-2ROD Vpx screen, one encoded aa 1–892 of α-actinin 1, two clones encoded aa 316–892 of α-actinin 1 and three clones encoded aa 346–892. To summarize, two independent clones of α-actinin 1 were identified in the SIVmac239 screen and three independent clones were identified in the HIV-2ROD screen, indicating sufficient complexity of the cDNA library and the specificity of the interaction with 239Vpx and HIV-2ROD Vpx. In order to quantify the interaction of the different α-actinin 1 clones and the two different Vpx proteins, liquid β-Gal assays were performed by using the yeast strain Y190. All α-actinin 1 clones, full-length as well as the N-terminal deletion variants, showed a strong, specific interaction with SIVmac239 Vpx and HIV-2ROD Vpx (Fig. 2).

Coprecipitation analyses confirm the interaction of α-actinin 1 with SIVmac239 Vpx and HIV-2ROD Vpx

To verify the interaction of HA–α-actinin 1 with SIVmac239 Vpx and HIV-2ROD Vpx, we performed binding assays in a mammalian system. For this purpose, pCMV6M 239vpx and pCMV6M RODvpx and the N-terminally HA-tagged α-actinin 1 (aa 346–892) were expressed in COS7 cells. Expression of the N-terminally Myc-tagged Vpx proteins and of HA–α-actinin 1 was confirmed by Western blot (Fig. 3b, c, e, f). For immunoprecipitation, whole-cell lysates were incubated with anti-Myc mAbs and precipitated proteins were separated by SDS-PAGE. The presence of HA–α-actinin 1 in the Myc–Vpx immune complexes was detected by immunoblot using anti-HA antibodies. Specific coimmunoprecipitation of HA–α-actinin 1 was detected readily with Vpx from SIVmac239 and HIV-2ROD, but not with mock-transfected cells (Fig. 3a, d). Comparable results were obtained by using full-length α-actinin 1 (Fig. 3g–l) and by immunoprecipitation for α-actinin 1 using anti-HA antibodies followed by Western blotting for Vpx (data not shown). These results indicate that HA–α-actinin 1 binds strongly to 239Vpx, as well as to RODVpx.

Vpx proteins of SIVmac239 and HIV-2ROD colocalize with α-actinin 1 in eukaryotic cells

It has been shown that Vpx proteins of SIV and HIV-2 localize to multiple subcellular compartments. In order to perform colocalization studies with α-actinin 1, we analysed the subcellular distribution of 239Vpx alone. In all examined cells, 239Vpx and RODVpx were detected in the nucleus or localized to the cytoplasm (Fig. 4a–d). COS7 cells that were transfected with the different HA–α-actinin 1 plasmids

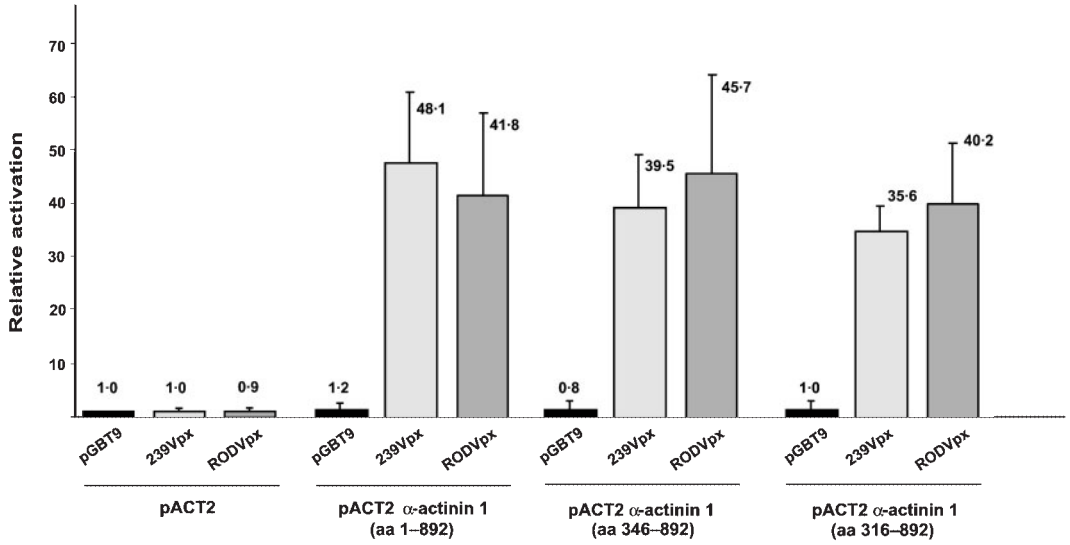


Fig. 2. Interaction of α-actinin 1 with the Vpx proteins from SIVmac239 and HIV-2ROD in yeast. Yeast cells were transformed with the indicated plasmids, selected in the absence of tryptophan and leucine and analysed for β-Gal activity. As controls, the Gal4 DNA-binding domain expression plasmid (pGBT9), 239Vpx and RODVpx expression plasmids were cotransformed with the empty pACT2 vector encoding the Gal4 activation domain. Additionally, pGBT9 was cotransformed with both pACT2 α-actinin 1 plasmids (encoding aa 1–892 and 316–892, respectively). β-Gal units were measured by analysing independent transformants and mean values were determined. Relative activation was calculated in relation to the vector control (pACT2/pGBT9) and is given in arbitrary units. Numbers above each bar represent mean values of five independent experiments; SD is indicated.

(encoding aa 346–892 and full-length α-actinin 1) showed an even distribution of HA–α-actinin 1 throughout the nucleus and the cytoplasm after staining with FITC-conjugated anti-HA antibodies (Fig. 4e and data not shown). Specificity of the antibodies used was shown by staining cells that were transfected with empty expression vectors (Fig. 4f). In order to test whether 239Vpx and RODVpx affect the subcellular distribution of α-actinin 1, COS7 cells were cotransfected with HA–α-actinin 1 (aa 346–892) and the Myc-tagged Vpx proteins. In contrast to cells that were transfected with the HA–α-actinin 1 expression plasmids alone, the cytoplasmic distribution of HA–α-actinin 1 in the presence of 239Vpx or RODVpx either decreased, whereas the accumulation of HA–α-actinin 1 in the nucleus became more pronounced (Fig. 4g, i, j), or both proteins (239Vpx/RODVpx and HA–α-actinin 1) accumulated at the nuclear membrane (Fig. 4h). Superimposition of the two confocal images clearly shows a high degree of colocalization and that 239Vpx and RODVpx influence the subcellular distribution of HA–α-actinin 1. Comparable results were obtained when the HA–α-actinin 1 full-length expression plasmids were used (data not shown).

C-terminal proline-rich domain of SIVmac239 Vpx is required for interaction with α-actinin 1

In order to map the binding domain of α-actinin 1 within Vpx, three N-terminal and three C-terminal deletion mutants of SIVmac239 *vpx* were generated (Fig. 5). The deletions are based on amino acid alignments between the highly homologous Vpx proteins of SIVmac239 and HIV-2ROD (Fig. 1) and earlier studies that defined three distinct structural domains within Vpx, two putative α-helices (I and II) and one proline-rich domain at the C terminus. All deletion mutants were subcloned into the yeast expression vector pGBT9 and expressed as hybrid proteins with the Gal4 DNA-binding domain. Expression of the deletion variants of 239Vpx in yeast was verified by Western blot analysis (data not shown). In order to perform interaction studies, SIVmac239 *vpx* deletion variants and α-actinin 1 (aa 346–892) were introduced in the yeast strain Y190. Subsequently, liquid β-Gal assays were performed on Leu^+ Trp^+ transformants (Fig. 6a). None of the C-terminal deletion variants showed an interaction with HA–α-actinin 1. Deletion of the C-terminal 11 aa, containing the proline-rich motif, was sufficient to abolish binding of 239Vpx to HA–α-actinin 1. In contrast, all three N-terminal deletion mutants exhibited a strong interaction with HA–α-actinin 1, whereas 239Vpx ΔN2 and 239Vpx ΔN3 showed a slightly stronger interaction with α-actinin 1 than the wild-type protein (Fig. 6a). None of the deletion mutants had an activating effect in the absence of α-actinin 1 (Fig. 6b). These results indicate that aa 102–112 of Vpx are required for binding to α-actinin 1.

To verify the results that were obtained in the yeast system, the six 239*vpx* deletion variants, as well as wild-type 239*vpx*, were fused to the GST-encoding gene. Expression of GST fusion proteins was verified by Coomassie staining of

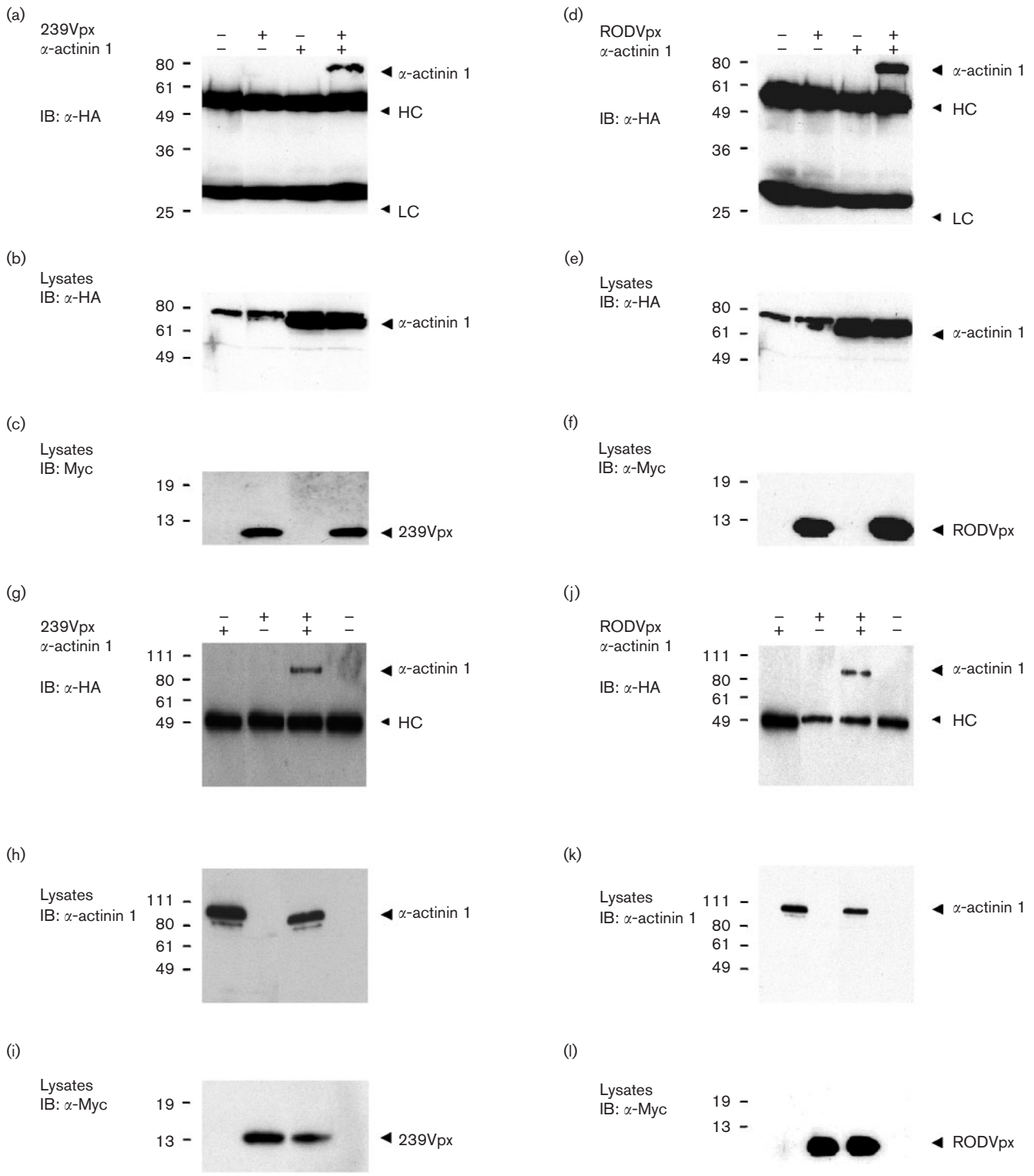


Fig. 3. Interaction of SIVmac239 (a–c, g–i) and HIV-2ROD (d–f, j–l) Vpx with α-actinin 1 in mammalian cells. COS7 cells were transfected with 5 μg pCMV6M 239vpx, pcDNA3HA–α-actinin 1 or both, as indicated. Mock transfection with pcDNA3 was also included (a, d, g, j). Immunoprecipitations were performed by using the anti-Myc mAb 9E10. Immunoprecipitated proteins were analysed by immunoblotting with anti-HA antibody. Arrowheads on the right of each immunoblot mark the heavy (HC) and light (LC) chains of the antibodies. Expression levels of α-actinin 1 (b, e, h, k), SIVmac239Vpx (c, i) and HIV-2ROD Vpx (f, l) were analysed in whole-cell lysates by immunoblotting (IB) using anti-HA antibodies (b, e), anti-α-actinin antibodies (h, k) and anti-Myc antibodies (c, f).

SDS–polyacrylamide gels (Fig. 6d). When purified GST proteins were mixed with COS7 cell lysates containing HA-tagged α-actinin 1, GST-239Vpx wild-type fusion protein bound efficiently to HA–α-actinin 1, whereas GST protein alone did not (Fig. 6c). In accordance with the results from the yeast system, all three N-terminal deletion mutants showed a strong interaction with HA–α-actinin 1, whereas none of the C-terminal deletion variants bound to HA–α-actinin 1. This proves that aa 102–112 are necessary for the interaction of 239Vpx and HA–α-actinin 1 and that deletion of the proline-rich motif is sufficient to abolish binding of 239Vpx to α-actinin 1 (Fig. 6c). In contrast to the ONPG assays, the *in vitro* GST-binding assays did not indicate an increased binding affinity for 239Vpx ΔN2 or 239Vpx ΔN3, as observed in the yeast system. In summary, these data show that HA–α-actinin 1 interacts with the Vpx proteins of SIVmac239 and HIV-2ROD and that the interaction of SIVmac239 and α-actinin 1 can be mapped to the C terminus of 239Vpx.

DISCUSSION

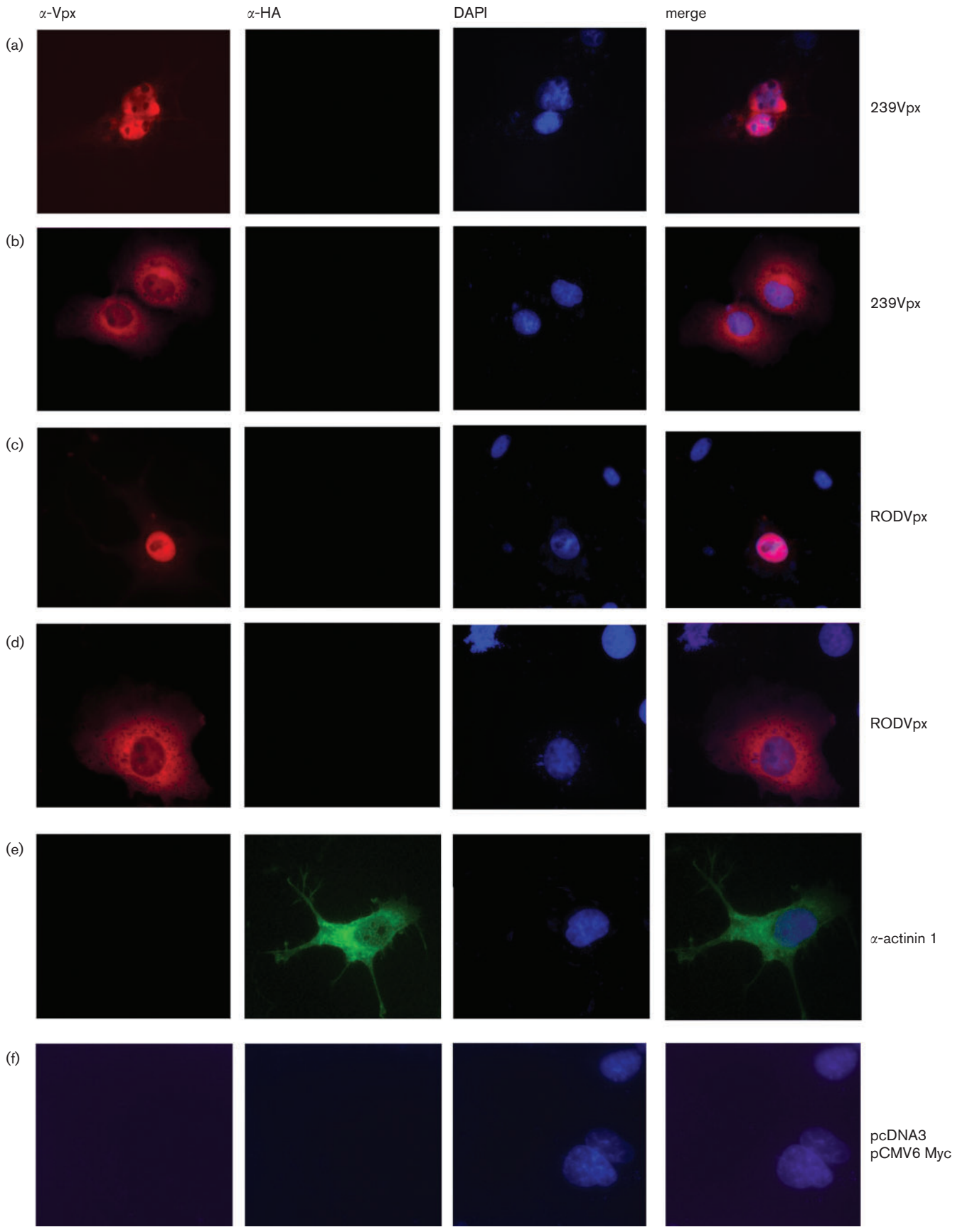
Intracellular pathogen-transport processes have been shown to be associated with components of the cytoskeleton (Bearer & Satpute-Krishnan, 2002; Sodeik, 2000). Bacterial actin-based motility is one of the best-documented examples of exploitation of mammalian cell machinery by bacterial pathogens such as *Listeria* and *Shigella* (Cossart, 2000). Furthermore, cytoskeleton-dependent transport has been shown for a number of different viruses. For example, it has been demonstrated that microtubules play a prominent role in efficient nuclear targeting during the entry of herpesviruses (Döhner *et al.*, 2002; Mabit *et al.*, 2002; Sodeik *et al.*, 1997) and adenoviruses (Mabit *et al.*, 2002; Suomalainen *et al.*, 1999). A recent study showed that, after viral entry, HIV particles move along the cytoskeleton to transport the viral PIC to the nucleus (McDonald *et al.*, 2002). This finding suggests a vital role for interaction of components of the viral PIC with proteins of the cytoskeleton. The PIC is a nucleoprotein complex that comprises a variety of viral components, including dsDNA and reverse transcriptase, matrix, nucleocapsid, integrase and Vpr (HIV-1) or Vpx (HIV-2, SIV) proteins (Bukrinsky *et al.*, 1993b; Farnet & Haseltine, 1991; Fletcher *et al.*, 1996; Fouchier & Malim, 1999; Hansen & Bushman, 1997; Heinzinger *et al.*, 1994; Miller *et al.*, 1997). These proteins have been demonstrated to be crucial regulators of nuclear PIC import, but it remains unclear as to the exact contribution that each protein makes. In the present study, we show that SIVmac239 and HIV-2ROD Vpx proteins interact with α-actinin 1 (Millake *et al.*, 1989), one of four different isoforms of a cytoskeletal protein that belongs to the spectrin superfamily (Dixson *et al.*, 2003). α-Actinin 1 and its isoforms interact with a large number of different cellular proteins, such as the cytoplasmic domains of several surface receptors (Carpén *et al.*, 1992; Heiska *et al.*, 1996; Pavalko & LaRoche, 1993; Pavalko *et al.*, 1995; Wyszynski *et al.*, 1997) and cell–cell adherence junction proteins (Craig & Pardo, 1979; Louis *et al.*, 1997; Schmeichel & Beckerle, 1994; Yamada & Geiger, 1997). Most importantly, they are involved in cytoskeletal reorganization (Critchley & Flood, 1999; Pavalko & LaRoche, 1993), bundling and cross-linking of actin filaments (Lazarides & Burridge, 1975; Podlubnaya *et al.*, 1975) and binding of actin to the plasma membrane (Takubo *et al.*, 1999).

It has been shown previously that Vpx augments HIV-2 replication in natural target cells by enhancing nuclear import of the viral genome (Ueno *et al.*, 2003) and is essential for PIC transport and replication in non-dividing macrophages (Pancio *et al.*, 2000). In order to elucidate the function of Vpx in the context of nuclear PIC transport, we performed yeast two-hybrid experiments to identify cellular targets of Vpx. Here, we show the interaction of Vpx with α-actinin 1 in yeast cells, as well as in mammalian cells. So far, this interaction has not been confirmed to occur in HIV-infectable cells. However, α-actinin 1 is expressed in T lymphocytes, indicating that the interaction with Vpx can occur *in vivo* (Egerton *et al.*, 1996).

When expressed alone, the localization of Vpx was concordant with its previously reported localization in the cytoplasm (Kappes *et al.*, 1993), nucleus (Depienne *et al.*, 2000; Di Marzio *et al.*, 1995; Mahalingam *et al.*, 2001; Pancio *et al.*, 2000) and nuclear membrane (Mahalingam *et al.*, 2001), but could not be detected at the plasma membrane (Kappes *et al.*, 1993). The lack of plasma-membrane staining is probably due to the absence of Gag proteins in transiently transfected cells. Further investigation is needed to determine whether the different localization patterns of Vpx proteins are relevant for PIC transport or are simply due to overexpression.

Interaction of the three different α-actinin 1 clones, one of them being full-length α-actinin 1, with Vpx was shown in the yeast two-hybrid screen and confirmed by liquid *β*-Gal assays. As the three different clones showed similar binding behaviour to 239Vpx and RODVpx in yeast cells, we reason that the binding domain of Vpx lies within aa 346–892 of α-actinin 1. The interaction of α-actinin 1 and the viral Vpx proteins was verified by coimmunoprecipitation assays and a clear colocalization of α-actinin 1 and Vpx in intracellular staining experiments. Remarkably, the localization patterns of Vpx and α-actinin 1 changed when coexpressed. In addition to the nuclear and cytoplasmic localization, distinct colocalization of both proteins was observed at the nuclear membrane. Takubo *et al.* (1999) showed that α-actinin 1 anchors actin filaments to the cell membrane. In concordance with these findings, it is conceivable that α-actinin 1 may be an early binding partner of the PIC following the uncoating process of the virus and may play a role during the transport of the PIC to the nucleus.

The binding domain of α-actinin 1 was mapped clearly to the C-terminal proline-rich region of SIVmac239 Vpx. N-terminal Vpx deletions did not interfere with the interaction of Vpx and α-actinin 1. Interestingly, a slight increase


α-Vpx
α-HA
DAPI
merge
(a)
239Vpx
(b)
239Vpx
(c)
RODVpx
(d)
RODVpx
(e)
α-actinin 1
(f)
pcDNA3
pCMV6 Myc

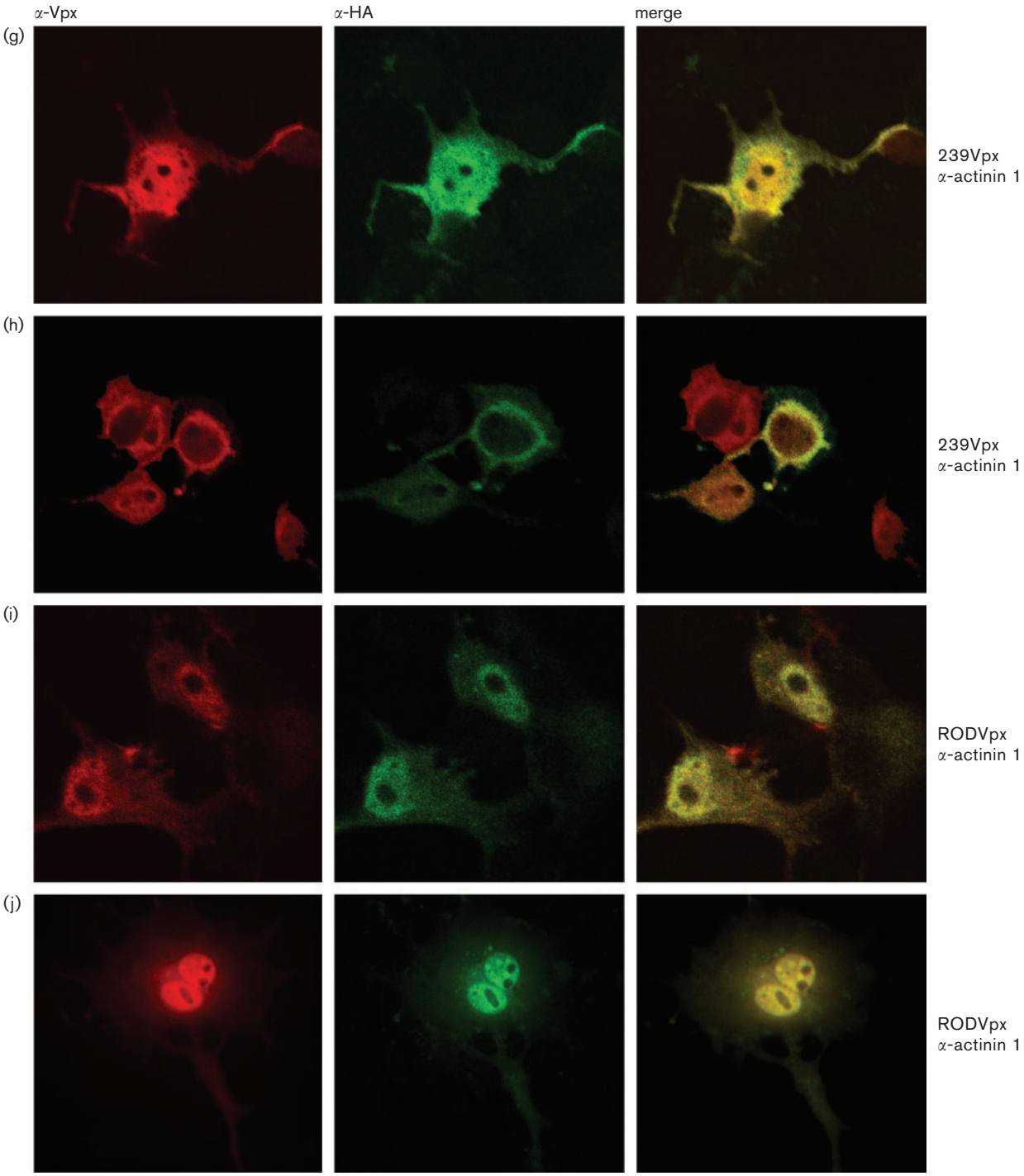


Fig. 4. Colocalization of α-actinin 1 and the Vpx proteins of SIVmac239 and HIV-2ROD. As controls, 1 μg pCMV6Myc expression plasmids for SIVmac239 Vpx and HIV-2ROD Vpx (a–d) or 1 μg pcDNA3 HA expression plasmid for α-actinin 1 (e) was introduced in COS7 cells. For colocalization analyses, cells were cotransfected with the pcDNA3 HA expression plasmid for α-actinin 1 and the pCMV6Myc expression plasmids for SIVmac239 Vpx and HIV-2ROD Vpx (g–j). Cells were fixed and stained 48 h after transfection. Vpx proteins (first column, shown in red) were detected by using a polyclonal anti-Vpx serum and Texas red-conjugated anti-rabbit secondary antibodies. α-Actinin 1 (second column, shown in green) was detected by using anti-HA antibodies and FITC-conjugated anti-mouse secondary antibodies. Nuclei were stained with DAPI (a–f). Fluorescence pictures were processed, anti-Vpx and anti-HA staining were overlaid with DAPI, and anti-Myc and anti-HA staining were overlaid (merge shown in yellow). No detectable background cross-staining was observed. For negative controls, cells were transfected with pcDNA3 and pCMV6 Myc (f); no detectable background cross-staining was observed.

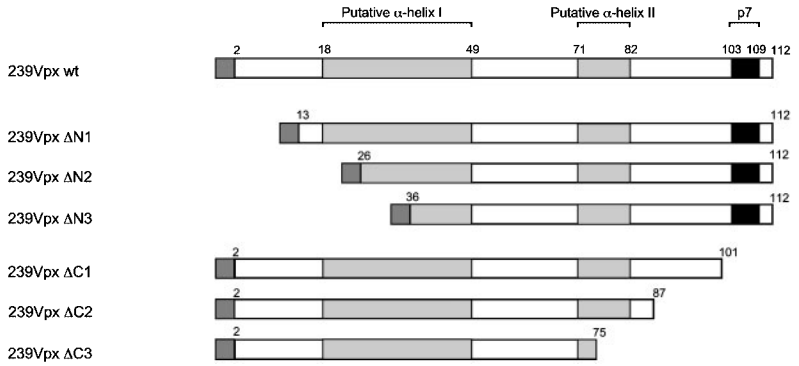


Fig. 5. Schematic representation of deletion mutations in SIVmac239 Vpx. Numbers indicate positions of amino acids in 239Vpx. The two putative α-helices (aa 18–49 and 71–82) are indicated as light grey boxes and the proline-rich domain p7 (aa 103–109) as a black box. The N-terminal deletion mutants were designated ΔN1 (aa 13–112), ΔN2 (aa 26–112) and ΔN3 (36–112); the C-terminal deletion mutants were designated ΔC1 (aa 2–101), ΔC2 (aa 2–87) and ΔC3 (aa 2–75).

(1·5–2·4-fold) in binding activity was observed for N-terminal deletion mutants, in comparison to wild-type Vpx, in the yeast system. This may be due to conformational changes of the protein, resulting in a higher binding affinity. So far, there are no data available concerning the three-dimensional structure or possible dimerization of Vpx, but data obtained for the homologous Vpr protein (Henklein *et al.*, 2000; Zhao *et al.*, 1994) indicate that self-association may influence the interaction of Vpx with cellular proteins. However, the mechanism of this effect remains unclear. So far, two new non-canonical NLSs have been identified within Vpx. The C-terminal proline-rich region of Vpx was reported by Pancio *et al.* (2000) to be important for Vpx-mediated nuclear import of the HIV-2

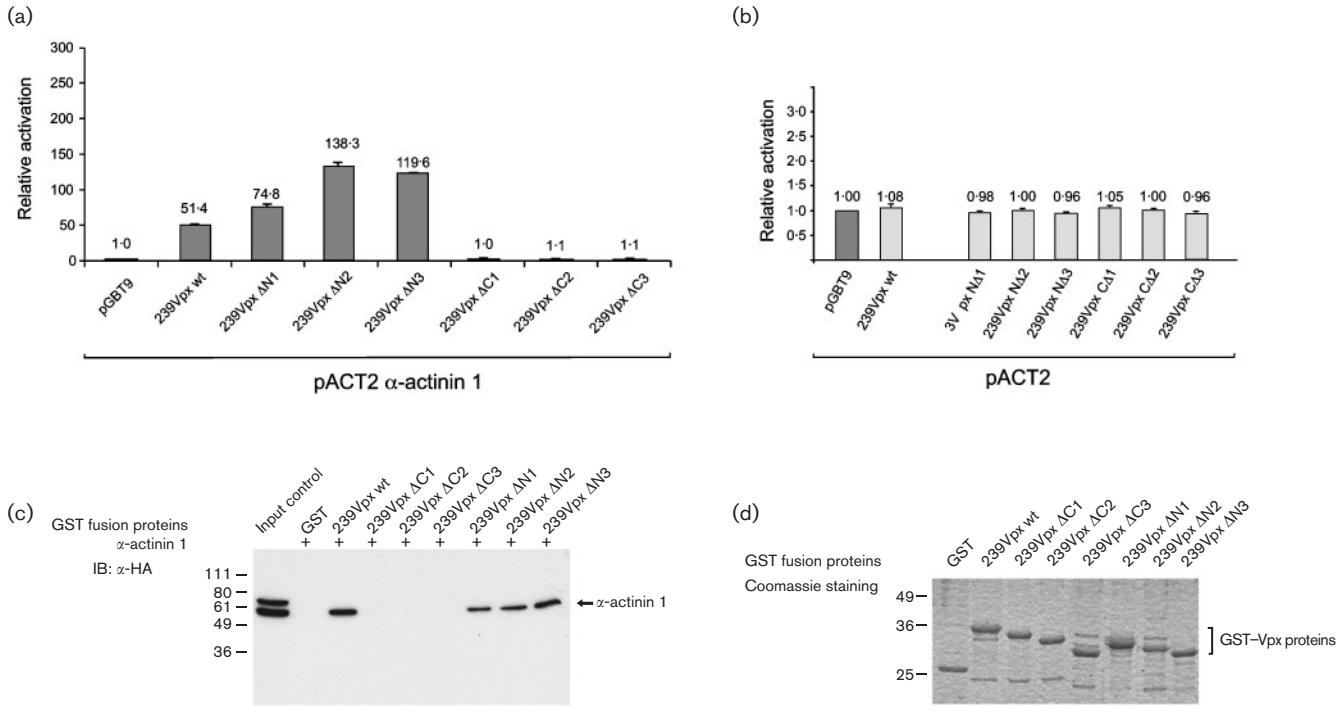


Fig. 6. C-terminal sequence motifs in SIVmac239 Vpx are necessary for interaction with α-actinin 1. (a, b) Yeast cells were transformed with pACT2 α-actinin 1 and one of the 239Vpx deletion mutant expression plasmids or the empty expression vector pGBT9. β-Gal activity was measured and mean β-Gal units were determined. Relative activation was calculated in relation to the vector control [pGBT9/pACT2 α-actinin 1 (a) or pGBT9/pACT2 (b)]. Numbers above each bar represent mean values of five independent transformants; SD is indicated. (c) GST-binding assay. Five micrograms of purified GST-SIVmac239 Vpx (239Vpx wt) and GST–239Vpx mutant fusion proteins (239Vpx ΔN1, 239Vpx ΔN2, 239VpxΔN3, 239Vpx ΔC1, 239Vpx ΔC2 and 239Vpx ΔC3) were incubated with equal amounts of HA-tagged α-actinin 1 expressed in COS7 cells and glutathione–Sepharose beads. Affinity-purified proteins were analysed by immunoblot (IB) using anti-HA mAbs. The input control contains 10 % of the α-actinin 1 protein that was used in each binding reaction. (d) Coomassie staining of 5 μg purified GST–239Vpx and GST–239Vpx mutant proteins that were used for affinity purification. Molecular mass marker is indicated on the left.

PIC. In the present study, we have shown that the C-terminal proline-rich domain of SIVmac239 Vpx is essential for its interaction with α-actinin 1. As the amino acid sequences of the Vpx proteins are highly conserved and the interaction of α-actinin 1 with SIVmac239 Vpx and HIV-2ROD Vpx is comparable, it is likely that binding of α-actinin 1 is required for Vpx-mediated nuclear import of the PICs of both viruses.

A second motif conferring nuclear localization has been described within Vpx between aa 60–85 and 65–72 (Belshan & Ratner, 2003; Kumar *et al.*, 2003; Mahalingam *et al.*, 2001). Considering that our data suggest that the C-terminal deletion was sufficient to abolish α-actinin 1 binding, these authors' results may define additional structural requirements for Vpx-mediated nuclear import. Our findings imply that Vpx tethers the PIC to the cytoskeleton via α-actinin 1 and may therefore play an important role in transport of the viral PIC.

The second NLS, and possibly interaction with additional cellular proteins, may be necessary for transfer of the PIC through the nuclear membrane. Consistent with published data (Pancio *et al.*, 2000), our results provide strong evidence for the existence of an α-actinin 1-dependent pathway of Vpx transport and associated transport of the viral PIC.

As nuclear transport of Vpx and transport of the PIC become increasingly complex, further investigations of the interaction between viral and cellular proteins with Vpx and other compounds of the PIC will be required to clarify the mechanism of nuclear import of the viral genome. Precise knowledge of the mechanism by which the PIC is imported into the nucleus could provide new means to intervene in the viral life cycle before integration of the viral genome into the host DNA takes place.

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