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HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import

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Human immunodeficiency virus type-1 (HIV-1) is able to infect non-dividing cells such as tissue macrophages productively because post-entry viral nucleoprotein complexes are specifically imported into the nucleus in the absence of mitosis. Although it has been proposed that an amino-terminal region of the viral matrix (MA, p17^{Gag}) protein harbors a basic-type nuclear localization sequence (NLS) that contributes to this process, utilization of three distinct nuclear import assays failed to provide any direct supporting evidence. Instead, we found that disruption of this region (^{26}KK→TT) reduces the rate at which the viral Gag polyprotein (p55^{Gag}) is post-translationally processed by the viral protease. Consistent with the fact that appropriate proteolytic processing is essential for efficient viral growth in all cell types, we also show that the ^{26}KK→TT MA mutation is equivalently deleterious to the replication of a primary macrophage-tropic viral isolate in cultures of non-dividing and dividing cells. Taken together, these observations suggest that proteins other than MA supply the NLS(s) that enable HIV-1 to infect non-dividing cells.

Keywords: Gag processing/HIV-1/macrophages/matrix protein/nuclear import

Introduction

Human immunodeficiency virus type 1 (HIV-1) and other lentiviruses replicate efficiently in both non-dividing (post-mitotic) and dividing cells (Weinberg *et al.*, 1991; Lewis *et al.*, 1992). Importantly, HIV-1 infection of non-dividing cell populations *in vivo*—and in particular of terminally differentiated tissue macrophages and mucosal dendritic cells—appears to be essential for initiating a pathogenic infection and for establishing viral reservoirs that can persist for extended periods (Gartner *et al.*, 1986; Koenig *et al.*, 1986; Wiley *et al.*, 1986; Schuitemaker *et al.*, 1992; Zho *et al.*, 1993; Connor and Ho, 1994; van't Wout *et al.*, 1994; Cornelissen *et al.*, 1995; O'Brien and Pomerantz, 1996). The ability of HIV-1 to infect non-dividing cells productively contrasts with the finding that replication of the oncogenic retrovirus murine leukemia virus (MLV) is restricted to dividing (proliferating) cells (Roe *et al.*, 1993; Lewis and Emerman, 1994). This key difference between the life cycles of these two retroviruses has been attributed to the ability of the post-entry viral nucleoprotein complexes (frequently termed pre-integration complexes or PICs) of HIV-1 to be actively imported into the nucleus during interphase (Bukrinsky *et al.*, 1992). In contrast, it has been demonstrated that the PICs of MLV only gain access to the nuclear interior following mitotic nuclear membrane dissolution (Roe *et al.*, 1993).

The trafficking of molecules between the nucleus and the cytoplasm occurs through gated channeled structures known as nuclear pore complexes (NPCs) (Feldherr *et al.*, 1984; Dworetzky and Feldherr, 1988). Because the size limit for diffusion through NPCs is ~50 kDa, molecules or complexes in excess of this require both a specific targeting signal and energy utilization for transport to take place (Hinshaw *et al.*, 1992; Davis, 1995; Görlich and Mattaj, 1996). Indeed, a number of distinct signals, receptor systems and pathways that mediate nuclear import have already been described (Izaurralde and Mattaj, 1992; Dingwall, 1996; Görlich and Mattaj, 1996). With respect to the import of many proteins, specific peptide motifs that serve as autonomous nuclear localization signals (NLSs) have been defined (Dingwall and Laskey, 1991; Garcia-Bustos *et al.*, 1991; LaCasse and Lefebvre, 1995). Typically (though not always), these sequences comprise one or two stretches of basic amino acids, the best known example is the NLS of simian virus 40 (SV40) T antigen (Figure 1). Interestingly, the import of multi-component nucleic acid–protein complexes such as the spliceosomal small nuclear ribonucleoprotein particles (U snRNPs) is more complicated. In particular, the nuclear uptake of U snRNPs is determined by a composite NLS that includes the 5′-trimethylated cap of the RNA as well as the Sm core proteins (Izaurralde and Mattaj, 1992; Fischer *et al.*, 1993).

By analogy with the mechanism of signal-mediated nuclear import and given that the size of the HIV-1 PIC resembles that of the ribosome (Farnet and Haseltine, 1990), one can expect that one or more of the components of the PIC will harbor NLSs that function during infection of non-dividing cells. To date, two HIV-1 encoded proteins, matrix (MA or p17^{Gag}) and Vpr, have been implicated as containing NLSs important for such infections (Bukrinsky *et al.*, 1993a; Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994; Gallay *et al.*, 1995a). In the case of MA, a putative basic-type NLS at residues 25–33 that bears some sequence similarity to the prototypic basic NLS of T antigen has been recognized (Figure 1). It was further shown that viruses carrying a double lysine to threonine substitution in this region (^{26}KK→TT) displayed levels of infectivity and replication that were diminished in non-dividing target cells but indistinguishable from corresponding wild-type viruses in dividing cells (Bukrinsky *et al.*, 1993a; Heinzinger *et al.*, 1994; von Schwedler *et al.*,

clade B consensus:	^{25}GKKkYkLKH
HXB/LAI:	GKKKYKLKH
YU-2:	GKKQYRLKH
SV40 T antigen:	PKKKRKV

Fig. 1. Amino acid sequence alignment of the amino-terminal basic region of HIV-1 MA (residues 25 to 33) with the defined NLS of SV40 large T antigen. The consensus sequence for clade B isolates is shown together with the precise sequences for HIV-1_{HXB}, HIV-1_{LAI} and HIV-1_{YU-2}.

1994). In contrast, one group has reported that a ^{26}KK→TT MA mutant virus replicates only marginally less well than the wild-type virus in non-dividing, as well as dividing, cell populations (Freed *et al.*, 1995).

Importantly, what has been lacking in the majority of these studies has been the direct assignment of NLS function according to conventional criteria. Specifically, sufficiency in terms of conferring nuclear import on heterologous substrates that have relative molecular masses that are greater than ~50 kDa, and therefore exceed the limit for diffusion through NPCs, has not generally been demonstrated for components of the HIV-1 PIC. Accordingly, it is conceivable that the aforementioned mutation in the amino-terminal basic region of MA could perturb a step (or steps) in the HIV-1 life cycle that occurs prior to nuclear import but that the ultimate consequence is not manifested until later, namely during nuclear uptake itself. For instance, one could envision that the integrity of the MA basic domain might be important for establishing a PIC conformation that exposes a non-MA-encoded NLS to cellular nuclear import factors.

We have therefore been utilizing two complementary experimental strategies to dissect the nuclear import of HIV-1 PICs. First, we have been assigning and defining NLS function—in terms of sufficiency—for the virally encoded components of PICs by using experimental systems previously developed by various laboratories for the analysis of protein nuclear import. Second, we have been disrupting potential NLSs within infectious molecular clones and then determining the ensuing phenotypes not only in terms of overall replication but also with respect to specific aspects of the life cycle that could be affected by that mutation. Regarding this latter point, rather than restricting our analyses to a lymphocyte-tropic viral isolate, for example HIV-1_{HXB} (Shaw *et al.*, 1984), we have been focusing our studies on a naturally macrophage-tropic isolate, HIV-1_{YU-2} (Li *et al.*, 1992). Indeed, HIV-1_{YU-2} is a more logical choice for these experiments since our long-term goal is to understand viral infection of cells such as macrophages. For reasons that will become apparent, only data that pertain to the nuclear import capabilities and function of MA are discussed in this manuscript. In summary, we report not only that MA does not appear to possess an NLS, but also that disruption of the previously proposed basic-type NLS at residues 25–33 both inhibits virus replication equivalently in all cell types and reduces the rate at which the Gag polyprotein is processed.

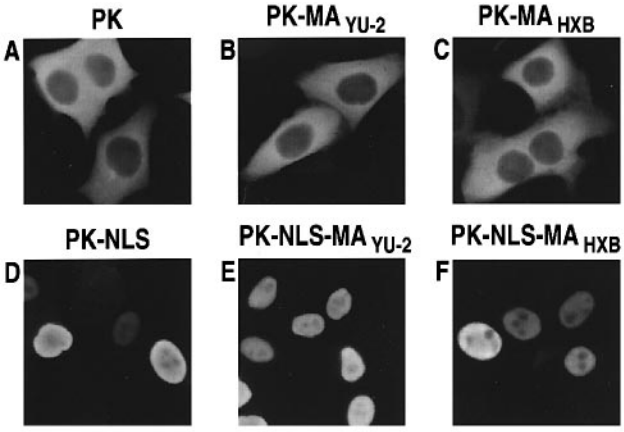

Fig. 2. Subcellular localization of pyruvate kinase (PK) fusion proteins in HeLa cells. Monolayer cultures were transfected with the indicated c-Myc epitope-tagged expression vectors and analyzed by indirect immunofluorescence using the Myc-specific monoclonal antibody.

Results

Evidence that the HIV-1 MA protein does not harbor an NLS

Here, we have utilized three different experimental approaches to evaluate NLS function in the HIV-1 MA protein. Importantly, each methodology has previously been used to demonstrate that a given peptide NLS can be sufficient for nuclear import. In each case, we have appended the 'test' protein (or peptide) to a heterologous substrate protein such that the final molecule is too large to diffuse (in either direction) through NPCs. According to this strategy, the failure of a given molecule to accumulate in the nucleus following its expression in (or introduction into) the cytoplasm is taken as evidence against that particular test sequence harboring a functional NLS.

For the first set of experiments, transfection-mediated expression of carboxy-terminal fusions to the 55 kDa cytoplasmic protein pyruvate kinase (PK) was used to examine the nuclear import of MA in somatic cells (Figure 2). The entire MA coding regions (132 amino acids) of both HIV-1_{YU-2} and HIV-1_{HXB} were therefore appended to PK, as was the 18 amino acid bipartite basic NLS of the human heterogeneous nuclear ribonucleoprotein particle (hnRNP) K protein which served as a positive control. Following transient transfection of HeLa cell monolayers, the subcellular localizations of the various chimeric proteins were determined by indirect immunofluorescence using a monoclonal antibody that recognizes an introduced epitope tag at the amino-terminus of PK. As anticipated, PK as a non-fusion protein was confined exclusively to the cytoplasm (Figure 2, panel A) whereas PK-NLS was efficiently imported into the nucleus (panel D). In sharp contrast, and unexpectedly, both PK-MA fusion proteins were localized entirely to the cytoplasm (Figure 2, panels B and C). To investigate the possibility that the amino-terminal basic domains (residues 25–33) of MA were somehow masked in these fusions, the previously defined K protein NLS (Michael *et al.*, 1995) was inserted between the PK and MA sequences. Both resulting PK–NLS–MA fusions were targeted to the nucleus (Figure 2, panels E and F), thus arguing against NLS occlusion being the reason for the cytoplasmic

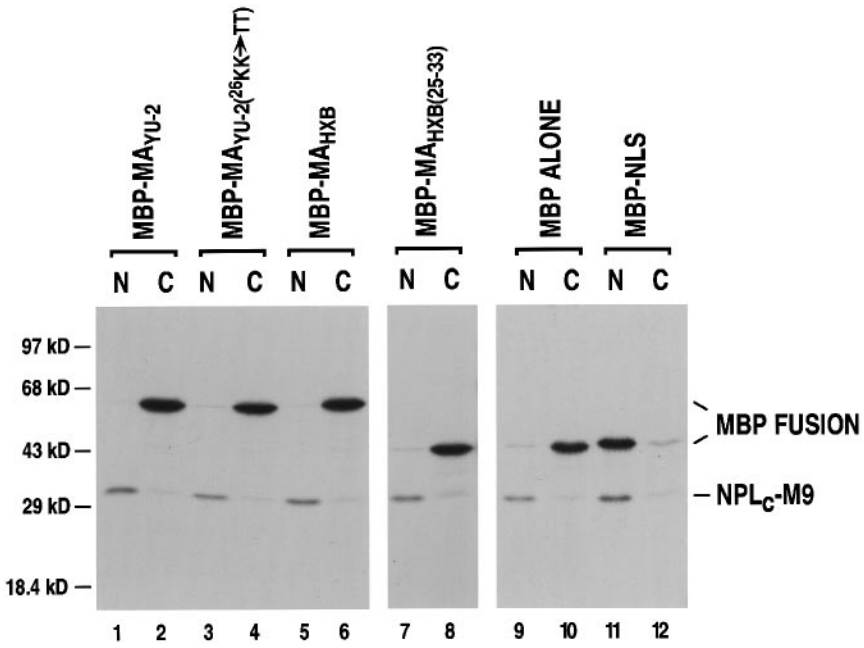

Fig. 3. Nuclear import of maltose binding protein (MBP) fusion proteins in microinjected *Xenopus laevis* oocytes. MBP fusion proteins and NPL_C-M9 were radiolabeled *in vitro* and co-injected into the cytoplasms of 10–20 stage VI oocytes. At 12 h, the oocytes were separated into nuclear (N) and cytoplasmic (C) fractions and the soluble proteins analyzed on a 12% SDS–polyacrylamide gel.

localization of the PK–MA fusions. To verify that these observations were not a consequence of placing MA at the carboxy-terminus of PK, we also appended a myristoylation-defective version of MA ($^{2}G\rightarrow A$) to the amino-terminus of β-galactosidase (β-GAL). The inhibition of myristoylation was critical for this experiment as this amino-terminal acylation mediates the targeting of Gag to the plasma membrane as a prerequisite to viral assembly (Göttlinger *et al.*, 1989; Wang and Barklis, 1993; Spearman *et al.*, 1994; Zhou *et al.*, 1994). The finding that the ~120 kDa MA–β-GAL fusion failed to accumulate in the nucleus further suggested that HIV-1 MA does not harbor an NLS (data not shown).

As a second approach for NLS definition, the nuclear import potential of MA was next evaluated by microinjecting maltose binding protein (MBP) fusions into *Xenopus laevis* oocytes. Accordingly, the full-length MA proteins of HIV-1_{YU-2} and HIV-1_{HXB}, the $^{26}KK\rightarrow TT$ mutant derivative of MA_{YU-2}, residues 25 to 33 of MA_{HXB} and the NLS of K were each translated *in vitro* as radiolabeled fusions to the carboxy-terminus of MBP. All five proteins, in addition to MBP as a non-fusion, were directly injected into the cytoplasms of stage VI *Xenopus* oocytes and transport to the nucleus assessed 12 h later by manual dissection into nuclear and cytoplasmic fractions followed by analysis on an SDS–polyacrylamide gel (Figure 3). As an internal positive control, all samples were co-injected with the nucleoplasmin core domain fused to the NLS of the hnRNP A1 protein (termed NPL_C-M9) (Siomi and Dreyfuss, 1995). As predicted, NPL_C-M9 and MBP-NLS efficiently accumulated in the nucleus (Figure 3, lanes 1, 3, 5, 7, 9 and 11) whereas MBP did not (lane 10). In complete agreement with the data obtained with PK fusions in somatic cells, the MBP fusions carrying full-length wild-type MA sequences both remained in the cytoplasm (Figure 3, lanes 1, 2, 5 and 6); not surprisingly, the $^{26}KK\rightarrow TT$ mutated derivative was also excluded from the nucleus (lanes 3 and 4). In this experiment, the question of potential masking effects was addressed by using MBP-$MA_{HXB(25–33)}$. Once again, this MA basic region-bearing protein failed to display any detectable nuclear import activity (Figure 3, lanes 7 and 8).

As a third and final strategy for the examination of possible NLS function in MA, synthetic peptides that carried introduced amino-terminal cysteine residues and encompassed residues 25 to 33 of MA_{YU-2}, residues 25 to 33 of MA_{HXB}, residues 23 to 34 of MA_{HXB} or residues 23 to 34 of the $^{26}KK\rightarrow TT$ derivative of MA_{HXB} were generated. Each was covalently coupled to bovine serum albumin (BSA), a protein of 68 kDa, that had previously been fluorescently labeled with Cy-3. All four BSA–peptide adducts were directly microinjected into the cytoplasms of living HeLa cells together with FITC-labeled rabbit IgG; the latter serving as a control for cytoplasmic injection since its relative molecular mass of ~160 kDa precludes transport across the nuclear envelope (Fantozzi *et al.*, 1994; Meyer *et al.*, 1996). The positive and negative control peptides for this experiment were, respectively, the NLS of wild-type simian virus 40 (SV40) large T antigen and its $^{128}K\rightarrow T$ non-functional derivative (Kalderon *et al.*, 1984). Nuclear import was then determined after 30 min by comparing the localization of the BSA–peptide conjugate with that of IgG using double-label immunofluorescence (Figure 4). As expected, the wild-type T antigen NLS mediated efficient import (Figure 4, panel A) whereas the mutated sequence did not (panel B). Once again, all MA-derived sequences, whether wild-type or mutated, failed to display any discernible import ability (Figure 4, panels C, D, E and F) and therefore displayed staining patterns that were essentially indistinguishable from the inactive T antigen control (panel B) and the IgG controls (panels I, J, K and L). As an additional control to verify that the BSA–peptide conjugates that failed to accumulate in the nucleus were not, in fact,

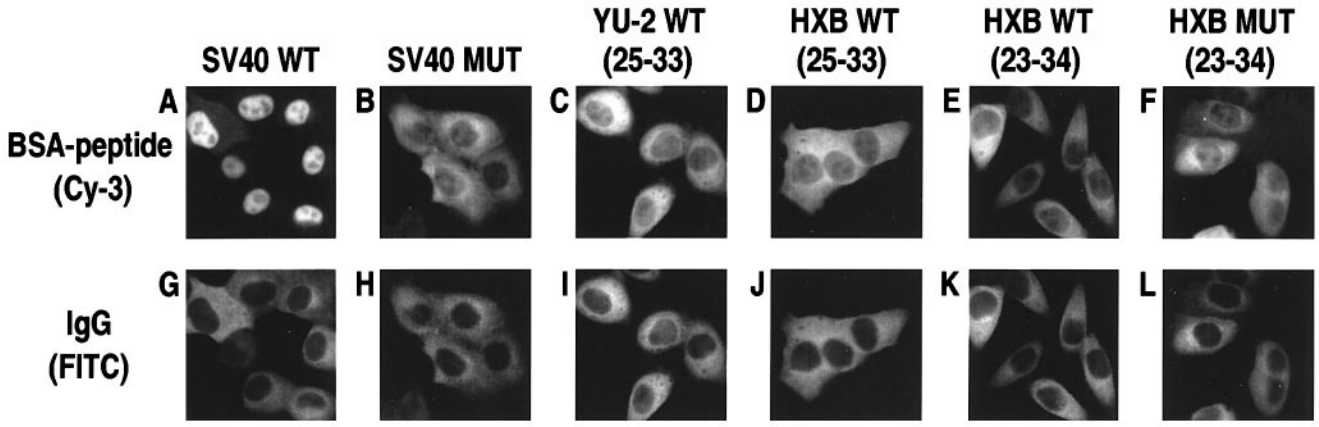

Fig. 4. Nuclear import of bovine serum albumin (BSA)–peptide adducts in microinjected somatic cells. Cy-3-labeled BSA–peptide conjugates were mixed with FITC-labeled rabbit IgG, co-injected into HeLa cell cytoplasms and visualized by double-label immunofluorescence at 30 min following fixation. Panels **A**–**F** show the localization of the BSA–peptide complexes, whereas panels **G**–**L** show the staining patterns for the IgG control in the same cells.

imported into the nucleus only to be rapidly exported back to the cytoplasm, some microinjections into nuclei were also performed. In these cases, the conjugates (as well as the IgG) remained confined to the nucleus for at least 60 min (data not shown).

In summary, we have exploited three distinct and complementary experimental approaches to investigate bona fide NLS function in the MA protein of HIV-1. In all cases, we have been unable to obtain any evidence to support this contention.

Importance of the amino-terminal basic domain of MA for HIV-1 replication and infection

In light of the above data and previous observations that HIV-1 harboring the ^{26}KK→TT MA mutation replicates poorly in non-dividing cells but as well as wild-type virus in dividing cells (Bukrinsky *et al.*, 1993a; Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994), it was clearly important to re-examine the replication and infection phenotypes of a ^{26}KK→TT MA mutant virus. This exact double-codon substitution was therefore introduced into an infectious proviral clone of HIV-1_{YU-2} and both mutant and wild-type virus stocks generated by transient transfection of 293T cells. The abilities of these two viruses to establish and maintain spreading infections were determined in monocyte-derived macrophages (MDMs, non-dividing cells) and primary blood lymphocytes (PBLs, dividing cells) derived from human peripheral blood as well as in the immortalized human T cell lines C8166-CCR5 and CEM-CCR5 (dividing cells) (Figure 5). Of note, these two T cell lines had first been rendered susceptible to HIV-1_{YU-2} infection by stable expression of the CCR5 chemokine receptor, a co-receptor that is utilized by this particular virus for entry (Choe *et al.*, 1996). Replication was monitored over time as the expression of soluble p24^{Gag} antigen in the culture supernatants. As can be readily visualized from all six viral challenges, the relationship between the replication profiles of the wild-type and MA mutant viruses was similar regardless of the proliferative status of the cells. Specifically, the MA mutant virus always replicated somewhat less efficiently than wild-type virus in the context of these spreading infections; this resulted in approximately one-tenth as much virus accumulation at most time points. These findings demonstrate that the ^{26}KK→TT MA mutation does not selectively inhibit replication of a primary macrophage tropic HIV-1 isolate in non-dividing cells. These results therefore stand in contrast to those obtained by

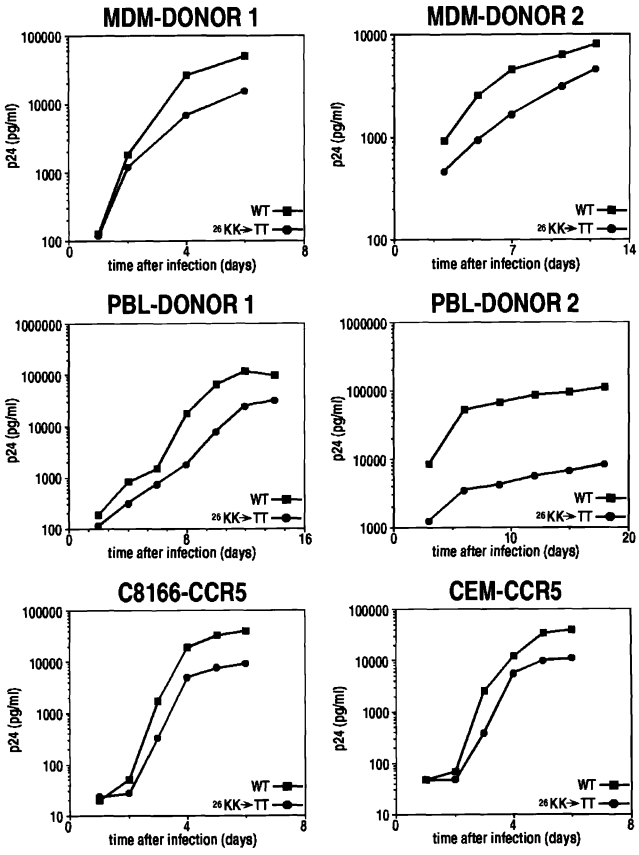

Fig. 5. Replication and phenotypes of ^{26}KK→TT MA mutant and wild-type HIV-1 in spreading infections of non-dividing and dividing cells. Monocyte-derived macrophages (MDMs), primary blood lymphocytes (PBLs), C8166-CCR5 and CEM-CCR5 cells were challenged with normalized stocks of HIV-1_{YU-2} produced by transfection of 293T cells. Virus production, and hence replication, was measured over time as the expression of soluble p24^{Gag} in the culture supernatants.

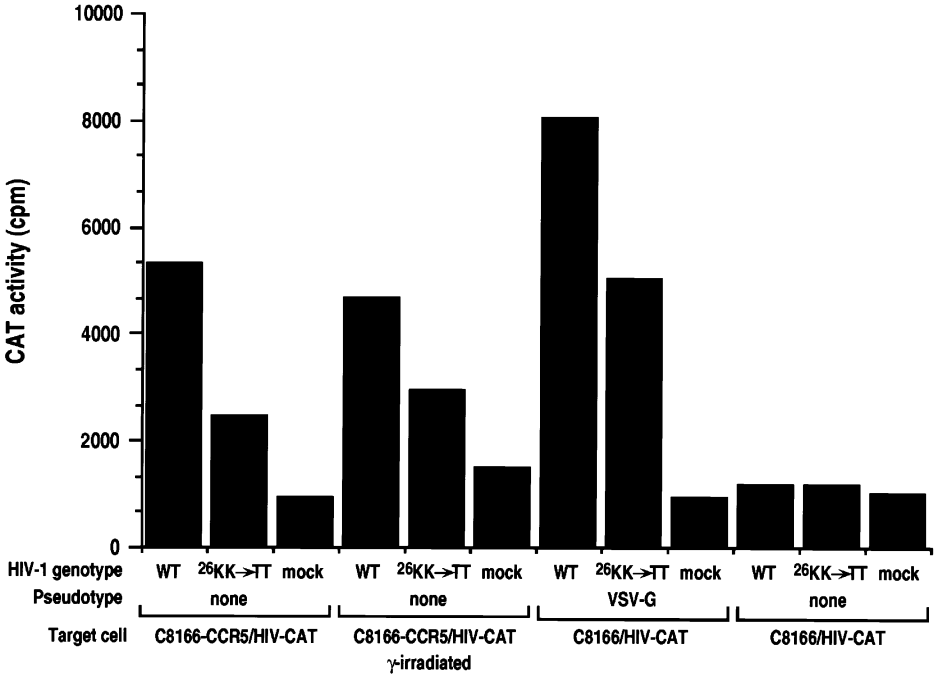

Fig. 6. Single-cycle infection phenotypes of ^{26}KK→TT MA mutant and wild-type HIV-1 in proliferating and arrested T cells. 5×10^5 C8166-CCR5/HIV-CAT or C8166/HIV-CAT cells were challenged with the same virus stocks used in Figure 5, with analogous stocks that had been pseudotyped by initial co-transfection with the pHIT/G expression vector or with medium containing no virus (mock) as indicated. At 24 h, cell lysates were prepared and the levels of CAT activity determined. The results of a representative series of challenges are shown. The relative infectivities of wild-type and MA mutant viruses were calculated after subtraction of the CAT values for mock infections.

some laboratories (Bukrinsky *et al.*, 1993a; Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994) but not others (Freed *et al.*, 1995).

To verify that the MA mutant HIV-1 was significantly less infectious than wild-type virus in single-cycle challenges, quantitations of infectivity were performed using T cell targets that were either proliferating or had been cell cycle-arrested (Figure 6). Here, the challenged cells harbored the chloramphenicol acetyl transferase (CAT) gene under the transcriptional control of the HIV-1 long terminal repeat (LTR) promoter element. As these cells became infected, the viral *trans*-activator Tat that was expressed by newly established proviruses not only activated transcription from those proviruses but also induced transcription from the resident HIV-CAT cassette. As a result, infection was quantitated as the induced accumulation of CAT within 24 h of viral challenge (Simon and Malim, 1996). In six independent infections of proliferating C8166-CCR5/HIV-CAT cells, wild-type HIV-1_{YU-2} was, on average, 2.5-fold more infectious than its ^{26}KK→TT MA mutant counterpart. Importantly, this differential in infectivity between this pair of viruses was maintained when the cells were rendered non-dividing by treatment with 4000 rad γ-irradiation 48 h prior to viral exposure. Thus, as with spreading infections (Figure 5), one-step measurements of viral infection demonstrated that the ^{26}KK→TT MA mutation is similarly inhibitory in challenges of dividing and non-dividing cells.

In an additional experiment, HIV-1 cores were pseudotyped with the envelope glycoprotein of the rhabdovirus vesicular stomatitis virus (VSV G) such that virus binding and entry occurred independent of gp120/41^{Env}, CD4 and CCR5. Interestingly, disruption of the MA basic region still reduced viral infectivity to a significant degree (Figure 6). This observation indicates, therefore, that the ^{26}KK→TT MA mutation likely affects at least one aspect of HIV-1 nucleoprotein core function.

The amino-terminal basic domain of MA is important for HIV-1 Gag processing

In an attempt to identify a possible biochemical explanation for the difference in infectivity between the cores of wild-type and ^{26}KK→TT MA mutant viruses, the patterns of Gag expression in virus-producing cells and budded virions were examined. Total cell lysates of cultures expressing wild-type and ^{26}KK→TT MA mutant HIV-1_{YU-2} and HIV-1_{LAI} proviruses, as well as lysates of viral particles purified from the matched culture supernatants, were prepared and analyzed by Western blotting using monoclonal antibodies specific for capsid (CA, p24^{Gag}) and MA (Figure 7). Strikingly, the profiles of Gag expression were very different between cultures expressing wild-type or MA mutant viruses. In particular, whereas the levels of total Gag expression were similar, the abundance of certain proteolytic processing intermediates (~39 kDa and ~25 kDa) as well as the mature, fully cleaved Gag proteins (most notably MA itself) were substantially reduced in the ^{26}KK→TT samples (Figure 7, compare lanes 2 and 4 with lanes 1 and 3). In contrast, the virion particle Gag proteins were processed to virtual completion in all wild-type and MA mutant samples (lanes 5–8). These findings are therefore very revealing as they demonstrate that the ^{26}KK→TT MA mutation reduces the rate at which Gag is post-translationally processed without affecting the ultimate extent (i.e. the endpoint) of cleavage. We speculate that it is the perturbation of normal Gag processing rates by the ^{26}KK→TT MA mutation that results in the generic

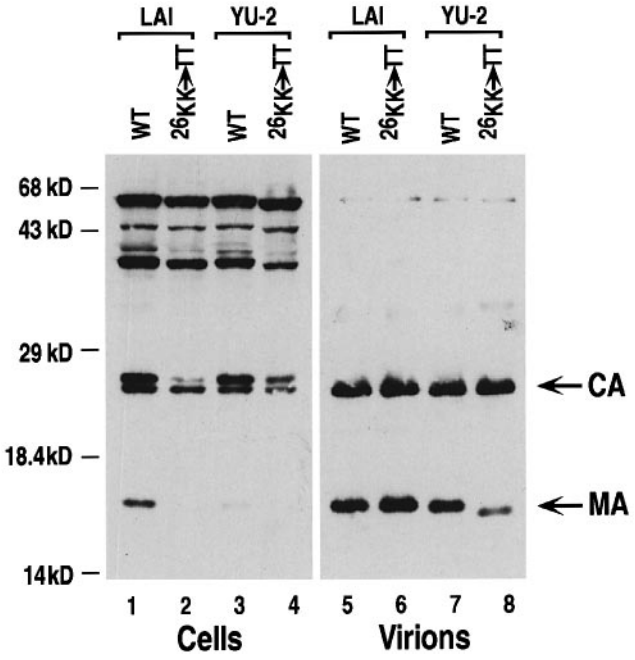

Fig. 7. Expression of HIV-1 Gag proteins in virus-producing cells and budded virions. Whole-cell lysates and purified viral particles derived from cells expressing wild-type and ^{26}KK→TT MA mutant, HIV-1_{LAI} or HIV-1_{YU-2}, proviral vectors were resolved by electrophoresis and subjected to Western analysis using Gag-specific monoclonal antibodies. The bands corresponding to the fully cleaved CA (24 kDa) and MA (17 kDa) proteins are indicated.

diminution of infectivity and replication noted for this virus (Figures 5 and 6).

Discussion

In this report, we have presented several distinct lines of evidence that address the controversial view that the amino-terminal region of HIV-1 MA harbors an NLS that directly mediates the nuclear import of viral PICs and is therefore selectively important for infection of non-dividing cells. First, we have used three well-established and characterized nuclear import assays to demonstrate that residues 25–33 of HIV-1 MA are not, by themselves, able to mediate the nuclear import of heterologous marker proteins. This finding was not only true for the full-length MA protein (Figures 2 and 3) but was also the case for synthetic peptides encompassing residues 23–34 (Figure 4). According to these criteria, we have concluded that the amino-terminal basic region of MA cannot be regarded as an NLS in the strictest sense of the definition. In fact, the observation that this region does not function as an autonomous NLS is, in retrospect, not so surprising since hydrophobic amino acids such as tyrosine (residue 29) are not usually located within basic-type NLSs (refer to Figure 1) (LaCasse and Lefebvre, 1995).

Second, we have utilized the same missense MA mutation (^{26}KK→TT) that has been used by others to evaluate the contribution of this region to HIV-1 infection and replication. In contrast with previous analyses where T cell line-adapted viruses (HIV-1_{HXB}) that had been converted to macrophage tropism by *env* gene substitutions were used, our experiments utilized a primary macrophage-tropic isolate, HIV-1_{YU-2}. We reproducibly found that the replication phenotype of the MA mutant virus was, relative to the wild-type virus, similar in cultures of non-dividing and dividing cells. Specifically, replication of the MA mutant virus was approximately one order of magnitude less efficient in MDMs, PBLs and immortalized T lymphoid lines (Figure 5). Importantly, these observations were further underscored by the demonstration that the MA mutant virus was also less infectious in single-cycle challenges of dividing and non-dividing T cell populations (Figure 6). Based on these findings, we have concluded that the defect in replication of a virus carrying the ^{26}KK→TT MA mutation appears to be a general one that is unrelated to the proliferative status of the target cells.

Third, we have identified a clear biochemical defect in ^{26}KK→TT MA mutant viruses that manifests itself during the final stages of the viral life cycle. Specifically, both for the HIV-1_{YU-2} isolate examined here and for the HIV-1_{LAI} isolate previously evaluated by others, we have found that missense mutation of the amino-terminal basic region of MA reduces the rate at which $p55^{Gag}$ polyprotein precursors are proteolytically processed by the viral protease (PR) (Figure 7). Because Gag processing rates can have significant effects on HIV-1 infectivity (Pettit *et al.*, 1994), our results strongly suggest that at least one aspect of the decreased replication of ^{26}KK→TT MA mutant viruses is attributable to less efficient processing of $p55^{Gag}$ by PR. The notion that the integrity of this region influences cleavage rates is consistent with both biochemical (Spearman *et al.*, 1994; Zhou *et al.*, 1994) and crystallographic (Hill *et al.*, 1996) studies which have indicated that this basic domain is important for the association of $p55^{Gag}$ with the plasma membrane during viral assembly and, by extrapolation, for the activation of PR through Gag dimerization (Katz and Skalka, 1994). Less efficient membrane binding would therefore result in reduced rates of Gag–Gag interaction, PR activation and (ultimately) Gag polyprotein processing.

A critical question that arises from these studies concerns why other groups have found differential replication phenotypes for MA basic domain mutant viruses in non-dividing versus dividing cells (Bukrinsky *et al.*, 1993a; Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994). One possible explanation for these discrepancies might be that isolates such as HIV-1_{HXB} and HIV-1_{LAI} that have been selected for highly efficient growth in T cell lines may be more tolerant of the ^{26}KK→TT MA mutation in high-titer infections of dividing T cells than primary isolates such as HIV-1_{YU-2}. Notably, experiments carried out with a highly pathogenic isolate of simian immunodeficiency virus (SIV_{SM} Pbj1.9) appear to be generally consistent with our view that MA does not harbor an NLS; here, disruption of the amino-terminal basic region of MA failed to yield a virus that was selectively impaired for replication in non-dividing cells (Fletcher *et al.*, 1996). It is also worth emphasizing that our studies have not addressed whether MA is involved in post-entry events distinct from nuclear import. In particular, it has been suggested that: (i) serine and tyrosine phosphorylation of MA is required for the release of PICs from the membrane following fusion (Bukrinskaya *et al.*, 1996); and (ii) that tyrosine phosphorylation is required for the association of MA with integrase (IN) and, therefore, for MA incorporation into PICs (Gallay *et al.*, 1995a,b).

The conclusion that the HIV-1 MA protein does not harbor an NLS that directly facilitates infection of non-dividing cells implies that other components of the PIC—namely, IN, reverse transcriptase (RT), nucleocapsid (NC), Vpr, viral nucleic acids or cellular factors (Bukrinsky *et al.*, 1993b; Gallay *et al.*, 1995b)—must contribute the NLS(s) that target this viral nucleoprotein complex for nuclear import. Indeed, Vpr may be one such protein since *vpr*-deficient viruses have been shown to display reduced replicative capacity in non-dividing cells (Balliet *et al.*, 1994; Heinzinger *et al.*, 1994; Connor *et al.*, 1995; Freed *et al.*, 1995) and Vpr fusion proteins are imported into the nuclei of both transfected HeLa cells and micro-injected *Xenopus* oocytes (data not shown). However, it is also probable that additional components contribute to PIC import as *vpr*-deficient viruses are still able to replicate to a significant degree in macrophages. In this regard, we speculate that the combined contributions of Vpr and other PIC constituents to nuclear transport may be somewhat analogous to the composite NLS that determines the import of U snRNPs (Izaurralde and Mattaj, 1992; Fischer *et al.*, 1993). Identifying and defining all the functional NLSs of HIV-1 PICs will therefore be critical for improving the understanding of how this virus infects cells of the monocyte/macrophage lineage *in vivo*.

Materials and methods

Molecular clones

A pcDNA1 (Clontech) -based eukaryotic expression vector that encodes pyruvate kinase (PK) with a c-Myc epitope tag (Evan *et al.*, 1985) at its amino-terminus and a polylinker at its carboxy-terminus has been described (Michael *et al.*, 1995). An analogous vector in which the coding region of maltose binding protein (MBP) was substituted for PK was also constructed. These two vectors served as the substrates for expressing PK–MA and MBP–MA fusion proteins. In all cases, PCR-amplified fragments corresponding to the entire coding region of MA, or shorter subfragments, were ligated into the parental vectors as *Xho*I–*Xba*I fragments. The HIV-1 templates for PCR amplification were the pIIIB (Hwang *et al.*, 1991) and pYU-2 (see below) provirus expression vectors; these express HIV-1_{HXB} and HIV-1_{YU-2}, respectively. For the PK–NLS and PK–NLS–MA vectors, an *Eco*RI–*Xho*I fragment that contained the 17 amino acid bipartite basic NLS of the hnRNP K protein (Michael *et al.*, 1995) was introduced at the carboxy-terminus of PK. Similarly, the same hnRNP K NLS-containing fragment was appended to the carboxy-terminus of MBP to create the MBP–NLS vector. The vector that expresses the nucleoplasmin core domain fused to the NLS of hnRNP A1 (NPL_C-M9) has been described (Michael *et al.*, 1995).

The wild-type HIV-1_{YU-2} provirus expression vector employed for these studies, pYU-2, was a derivative of the original proviral clone (Li *et al.*, 1992) in which the disrupted ATG initiation codon of the *vpu* gene had been repaired by site-directed mutagenesis. The ^{26}KK→TT MA mutation was introduced into pYU-2 by PCR-mediated site-directed mutagenesis and the entire amplified region (*Bss*HII–*Nsi*I; positions 707–1249) sequenced on both strands to ensure that no other mutations had been introduced. These DNA manipulations were performed on two independent occasions; the two MA mutant viruses had identical growth phenotypes (data not shown). The wild-type HIV-1_{LAI} and ^{26}KK→TT MA mutant vectors have been described (Heinzinger *et al.*, 1994); importantly, the region of MA that spans residues 25–32 is identical in amino acid sequence for HIV-1_{LAI} and HIV-1_{HXB} (refer to Figure 1).

The CCR5 cDNA encoding MLV-based retroviral vector, LP-M/CCR5, was constructed using a derivative of LN-M (Simon *et al*., 1995) in which the *neo* gene had been replaced with one that confers resistance to puromycin. The VSV G expression vector, pHIT/G, was derived from pHIT123 (Soneoka *et al*., 1995) by substitution of the MLV *env* gene with an *Eco*RI fragment from pJC119 (Rose and Bergmann, 1983) that carried the gene for the glycoprotein of VSV. The MLV *gag*, *gag-pol* expression vector pHIT60 has been described (Soneoka *et al*., 1995).

Cells and cell lines

The immortalized human cell lines HeLa, 293T, CEM-SS and C8166, as well as the modified C8166/HIV-CAT that contains the chloramphenicol acetyl transferase (CAT) gene under the transcriptional control of the HIV-1 long terminal repeat (LTR) promoter element, have been described (Meyer and Malim, 1994; Simon *et al.*, 1995; Simon and Malim, 1996). Retrovirus-mediated gene transfer was used to stably express the CCR5 chemokine receptor in CEM-SS, C8166 and C8166/HIV-CAT cells such that they were rendered susceptible to HIV-1_{YU-2} infection. To achieve this, a 100 mm diameter culture of 293T cells was transiently transfected with 10 μg each of LP-M/CCR5, pHIT/G and pHIT60 using calcium phosphate, cell-free viral supernatant harvested at 24 h and used to challenge target cultures. Stably transduced cell populations were selected and maintained in complete RPMI 1640 medium supplemented with 10% fetal bovine serum and either 200 (CEM-SS and C8166) or 500 (C8166/HIV-CAT) ng/ml puromycin.

Localization of pyruvate kinase fusion proteins by indirect immunofluorescence

35 mm diameter sub-confluent HeLa cell monolayers were transiently transfected with 5 μg of the indicated c-Myc epitope tagged expression vectors using calcium phosphate. At 24 h, the cells were fixed using paraformaldehyde, permeabilized and the staining pattern for PK determined by hybridization first with the Myc-specific monoclonal antibody 9E10 (Evan *et al.*, 1985) and then with a Texas red (TXRD)-conjugated goat anti-mouse secondary antibody (Meyer and Malim, 1994). The samples were viewed by epifluorescence using a Nikon microphot-SA microscope at a magnification of ×400.

Nuclear import of maltose binding protein fusions in Xenopus laevis oocytes

The MBP fusion proteins and NPL_C-M9 were synthesized *in vitro* and labeled with [^{35}S]methionine using a T7-reticulocyte lysate-coupled transcription–translation system (Promega Corp.). The reaction products were examined for integrity by SDS–polyacrylamide gel electrophoresis followed by fluorography (data not shown) and each MBP fusion mixed with NPL_C-M9 and injected directly into the cytoplasms of 10–20 stage VI oocytes. At 12 h, the oocytes were manually dissected into nuclear and cytoplasmic fractions and the soluble proteins analyzed by electrophoresis through a 12% SDS–polyacrylamide gel, fluorography and autoradiography (Fischer *et al.*, 1995).

Nuclear import of bovine serum albumin–peptide conjugates in HeLa cells

Fluorescently tagged BSA–peptide adducts were generated by direct labeling of BSA with Cy-3 (Amersham Corp.) followed by the covalent coupling of synthetic peptides that each carried an introduced amino-terminal cysteine using the heterobifunctional cross-linker sulfo-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce). Efficient coupling of all peptides (at least 10 peptides per BSA monomer) was confirmed by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining (data not shown). These complexes were concentrated to ~2 mg/ml in phosphate-buffered saline and injected into HeLa cell cytoplasms or nuclei together with rabbit IgG that had been directly labeled with fluorescein isothiocyanate (FITC; Molecular Probes) (Meyer *et al.*, 1996). After 30–60 min incubation at 37°C, the samples were fixed and visualized by double-label immunofluorescence at a magnification of ×400. The peptide sequences were: SV40 T antigen wild-type (residues 125–135), C-YPKKKRKVEDP; SV40 T antigen ^{128}K→T mutant (Kalderon *et al.*, 1984), C-YPKTKRKVEDP; MA_{YU-2} wild-type (25–33), C-GKKQYRLKH; MA_{HXB} wild-type (25–33), C-GKKKYKLKH; MA_{HXB} wild-type (23–34), C-PGGKKKYKLKHI; MA_{HXB} ^{26}KK→TT mutant (23–34), C-PGGTTKYKLKHI. All peptides were synthesized using an Advanced Chemtech MPS 396 synthesizer.

Primary cells, HIV-1 replication and single-step infections

Monocyte-derived macrophages (MDMs) and primary blood lymphocytes (PBLs) were derived from the peripheral blood of healthy volunteer donors following venepuncture. MDMs were purified by gelatin-coated plastic adherence (Collman *et al.*, 1989) and maintained in 24-well culture dishes at a density of 4×10^5 cells per well in DMEM supplemented with 20% fetal bovine serum and 100 U/ml recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) for 7 days prior to viral challenge. PBLs were purified using Ficoll–Paque, stimulated with 5 μg/ml phytohemagglutinin (PHA) for 72 h and maintained in RPMI 1640 medium containing 20% fetal bovine serum and 20 U/ml recombin-

ant interleukin-2 (rIl-2) at a density of ~1×10^6 cells/ml (Simon and Malim, 1996).

Stocks of wild-type and ^{26}KK→TT MA mutant HIV-1_{YU-2} were generated by transient calcium phosphate-mediated transfection of 100 mm diameter cultures of 293T. At 24 h, the supernatants were harvested and stored in aliquots at –80°C. 4×10^5 MDMs, 5×10^6 PBLs, 0.5×10^6 C8166-CCR5 cells or 0.5×10^6 CEM-CCR5 cells were challenged with wild-type or mutant stocks corresponding to 10 ng soluble p24^{Gag} (PBLs of donor number 2 were challenged with 100 ng p24^{Gag}), as determined by enzyme-linked immunosorbent assay (ELISA). Cultures were maintained by replenishing the culture media at 2- to 3-day intervals and virus production measured as the expression of soluble p24^{Gag} in the culture supernatants.

For the one-step infections of C8166-CCR5/HIV-CAT or C8166/HIV-CAT cells, cultures containing 0.5×10^6 cells were challenged with stocks of wild-type or ^{26}KK→TT MA mutant HIV-1_{YU-2} corresponding to 10 ng soluble p24^{Gag}, VSV G pseudotyped stocks corresponding to 10 ng soluble p24^{Gag} that had been derived by co-transfection of the proviral expression vectors with pHIT/G, or medium alone (mock infection). At 24 h post-infection, whole-cell lysates were prepared and the levels of CAT activity determined as described (Simon and Malim, 1996). In experiments where the C8166-CCR5/HIV-CAT cells were cell cycle-arrested, cultures were subjected to 4000 rad γ-irradiation from a ^{137}Cs source and transferred to fresh medium for 48 h prior to viral challenge. For these cells, this treatment typically resulted in a redistribution of cells from ~40% (G_0/G_1), ~34% (S), ~26% (G_2/M) to ~20% (G_0/G_1), ~4% (S), ~76% (G_2/M) as determined by propidium iodide staining and flow cytometry analysis (data not shown).

Analysis of viral Gag proteins

Total cell lysates and viral particles pelleted from culture supernatants by centrifugation at 26 000 *g* for 60 min at 4°C were derived from 293T cultures transiently transfected with wild-type and ^{26}KK→TT MA mutant, HIV-1_{LAI} or HIV-1_{YU-2} provirus expression vectors. The samples were normalized for the amount of p24^{Gag} in the culture supernatants (1.0 μg/ml, wild-type HIV-1_{LAI}; 1.4 μg/ml MA mutant HIV-1_{LAI}; 1.1 μg/ml wild-type HIV-1_{YU-2}; 0.64 μg/ml MA mutant HIV-1_{YU-2}), denatured and the proteins resolved on a 12% SDS–polyacrylamide gel before being electrophoretically transferred to nitrocellulose. The filter was hybridized initially with a cocktail of two mouse monoclonal antibodies specific for CA (p24^{Gag}) and MA and subsequently with a horseradish peroxidase-conjugated goat anti-mouse antibody. Bound antibody was visualized by enhanced chemiluminescence followed by autoradiography.

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