Efficient HIV-1 replication can occur in the absence of the viral matrix protein

Heide Reil, Anatoly A.Bukovsky¹,
Hans R.Gelderblom² and
Heinrich G.Göttlinger³

Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, and Department of Pathology, Harvard Medical School, Boston, MA 02115, USA and ²Robert Koch-Institut, Berlin, Germany

¹Present address: Cell Genesys, Inc., 342 Lakeside Drive, Foster City, CA 94404, USA

³Corresponding author
e-mail: Heinrich_Gottlinger@DFCI.harvard.edu

Matrix (MA), a major structural protein of retroviruses, is thought to play a critical role in several steps of the HIV-1 replication cycle, including the plasma membrane targeting of Gag, the incorporation of envelope (Env) glycoproteins into nascent particles, and the nuclear import of the viral genome in non-dividing cells. We now show that the entire MA protein is dispensable for the incorporation of HIV-1 Env glycoproteins with a shortened cytoplasmic domain. Furthermore, efficient HIV-1 replication in the absence of up to 90% of MA was observed in a cell line in which the cytoplasmic domain of Env is not required. Additional compensatory changes in Gag permitted efficient virus replication even if all of MA was replaced by a heterologous membrane targeting signal. Viruses which lacked the globular domain of MA but retained its N-terminal myristyl anchor exhibited an increased ability to form both extracellular and intracellular virus particles, consistent with a myristyl switch model of Gag membrane targeting. Pseudotyped HIV-1 particles that lacked the structurally conserved globular head of MA efficiently infected macrophages, indicating that MA is dispensable for nuclear import in terminally differentiated cells.

Keywords: cytoplasmic domain/envelope/HIV-1/matrix protein/virus replication

Introduction

HIV-1 morphogenesis is directed by the Gag polyprotein Pr55^{gag}, the precursor for the internal structural proteins of the mature virion (Hunter, 1994). The assembling particle buds through the plasma membrane and thereby acquires a lipid membrane enriched in the viral surface (SU) and transmembrane (TM) envelope (Env) glycoproteins. Processing of Pr55^{gag} during virus maturation yields the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins. MA forms a spherical shell directly underneath the lipid membrane of the mature virion, CA forms the characteristic conical core, and NC is complexed with the genomic RNA within the core (Hunter, 1994).

Within the context of Pr55^{gag}, MA provides an N-terminal plasma membrane targeting domain (Kräusslich and Welker, 1996). Myristylation of the MA domain at an N-terminal glycine is required for the efficient association of Pr55^{gag} with the plasma membrane and for virus particle formation (Göttlinger *et al.*, 1989; Bryant and Ratner, 1990). In addition, a highly conserved N-proximal cluster of basic residues in MA mediates binding to acidic membrane phospholipids (Zhou *et al.*, 1994). The three-dimensional structure of HIV-1 MA reveals a globular 'head' formed by four α -helices and a C-terminal α -helix which projects away from the core domain (Massiah *et al.*, 1994; Matthews *et al.*, 1994; Hill *et al.*, 1996). The globular head forms trimers in all known crystal forms, and N-proximal basic residues are found on the upper surface of the trimer where they would be appropriately positioned to interact with acidic phospholipid head groups (Hill *et al.*, 1996).

The membrane-binding signal in MA appears exposed in the context of Pr55^{gag}, but hidden in mature MA (Zhou and Resh, 1996). This may allow MA to dissociate from the membrane in order to assist in early steps of the replication cycle (Bukrinsky *et al.*, 1993; Gallay *et al.*, 1995a). In contrast with oncoretroviruses, HIV-1 can productively infect non-dividing cells, a process which requires the active transport of the viral preintegration complex through the nucleopore (Weinberg *et al.*, 1991; Bukrinsky *et al.*, 1992; Lewis *et al.*, 1992). HIV-1 preintegration complexes have been reported to contain several karyophilic components, including MA, Vpr and integrase (IN) (Bukrinsky *et al.*, 1993; Heinzinger *et al.*, 1994; Gallay *et al.*, 1997). In MA, a putative nuclear localization signal (NLS) was identified in the N-proximal basic cluster (Bukrinsky *et al.*, 1993). Viruses carrying substitutions in the MA NLS replicated efficiently in dividing but not in growth-arrested cells or terminally differentiated monocyte-derived macrophages (MDM), consistent with a role of MA in translocating the HIV-1 preintegration complex through the nuclear membrane (Bukrinsky *et al.*, 1993; Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994). It was further shown that phosphorylation on serine and tyrosine residues releases MA from the membrane, enabling it to perform its nuclear import function (Bukrinskaya *et al.*, 1996). In particular, phosphorylation of MA on a C-terminal tyrosine has been shown to be crucial for the nuclear import of the viral preintegration complex and for virus replication in MDM (Gallay *et al.*, 1995a,b). However, others have reported conflicting results that indicate that neither the N-proximal basic cluster nor the C-terminal tyrosine of MA contribute selectively to HIV-1 replication in MDM (Freed *et al.*, 1995, 1997; Fouchier *et al.*, 1997).

MA is essential for the incorporation of HIV-1 Env protein spikes into nascent viral particles (Yu *et al.*, 1992b;

Dorfman *et al.*, 1994). Small alterations throughout the globular core of HIV-1 MA blocked the incorporation of autologous Env protein, but not that of amphotropic murine leukemia virus (A-MLV), a widely divergent oncoretrovirus (Freed and Martin, 1995; Mammano *et al.*, 1995). A deletion which removed ~80% of MA still allowed the functional incorporation of A-MLV Env protein, and the pseudotyped mutant particles retained ~10% of wild-type infectivity during a single round of virus transmission (Wang *et al.*, 1993). Interestingly, second-site mutations which truncated the unusually long cytoplasmic tail of HIV-1 TM fully restored the incorporation of the autologous Env protein complex into HIV-1 MA mutants (Freed and Martin, 1995; Mammano *et al.*, 1995). Additionally, truncating the cytoplasmic tail restored the ability of mutants with small alterations in MA to replicate in MT4 cells (Mammano *et al.*, 1995), a human T cell line that supports HIV-1 replication in the absence of the cytoplasmic domain of TM (Wilk *et al.*, 1992).

We now report that efficient HIV-1 replication can be observed in the absence of the entire globular core of MA, provided that an Env incorporation defect is corrected by the removal of the cytoplasmic tail of TM. Furthermore, spontaneous mutations in CA and NC allowed efficient HIV-1 replication even if the entire MA protein was replaced by a peptide which provided a heterologous myristyl anchor for membrane attachment. MA-deleted viruses consistently exhibited an increased ability to release extracellular virus particles, arguing against a requirement for the globular domain of MA for stable membrane association. We also report that the globular head of MA, which harbors the putative MA NLS (Bukrinsky *et al.*, 1993), is dispensable for early steps of the HIV-1 replication cycle in MDM.

Results

Incorporation of truncated but not full-length Env in the absence of the globular core of MA

We and others showed previously that a block in HIV-1 Env incorporation caused by small deletions or single amino acid substitutions in the globular core of MA can be reversed by removal of the cytoplasmic domain of TM (Freed and Martin, 1995; Mammano *et al.*, 1995). To examine whether any part of the globular core of MA is required for the incorporation of C-terminally truncated HIV-1 Env, we deleted 119 of the 132 codons for MA from a proviral clone of HIV-1 (Figure 1). Surprisingly, despite the absence of most of MA, the resulting $\Delta 8-126$ mutant produced up to 4-fold more particles than the wild-type counterpart upon transfection into HeLa cells (Figure 2A). Immunoprecipitation from the cell lysates showed that the $\Delta 8-126$ mutation did not increase viral protein expression (data not shown), indicating that the deletion improved the efficiency of viral particle assembly or release. The protein composition of $\Delta 8-126$ mutant particles suggested that the large deletion still allowed efficient processing of the Gag polyprotein. As expected, $\Delta 8-126$ particles lacked MA, SU and TM, while these proteins were readily detectable in wild-type particles (Figure 2A).

To examine whether the mutant was able to incorporate C-terminally truncated HIV-1 Env, the $\Delta 8-126$ deletion

Fig. 1. Schematic representation of MA deletion mutants. The white and black boxes within the expanded view of MA indicate the positions of α -helices I–V and the two 3_{10} helices, respectively. Other shadings indicate heterologous sequences. The position of the Δ CT mutation, which introduces a premature termination codon into *env*, is also indicated.

was combined with the previously described Δ CT mutation, which essentially removes the long cytoplasmic tail of TM (Mammano *et al.*, 1995). As shown in Figure 2A, the levels of SU relative to CA in $\Delta 8-126/\Delta$ CT particles were at least as high as in wild-type virions. In addition, a significant amount of the C-terminally truncated Env precursor was incorporated. These results established that the entire globular core of MA is dispensable for the incorporation of C-terminally truncated HIV-1 Env protein.

Efficient virus replication in the absence of the globular core of MA

Previously, we observed that the Δ CT mutation can correct the replication defect caused by a three amino acid deletion in MA (Mammano *et al.*, 1995). To investigate whether larger deletions in MA can be tolerated, variants of the Δ CT mutant were generated which lack MA codons 36–57 or 36–87 (Figure 1). Equivalent amounts of virus produced in HeLa cells were used to infect MT4 cells. Virus replication was monitored over time by measuring reverse transcriptase (RT) activity in the culture supernatants. While the $\Delta 36-57/\Delta$ CT mutant did not yield a spreading infection, the $\Delta 36-87/\Delta$ CT mutant replicated, although with significantly delayed kinetics (data not shown).

The finding that the more extensive deletion appeared less disruptive prompted us to examine the effects of larger deletions. Remarkably, the removal of an additional 28 residues from the N-terminus of MA, which yielded

Fig. 2. Efficient Env incorporation and virus replication in the absence of the globular core of MA. (A) Restoration of Env protein incorporation through removal of the cytoplasmic domain of HIV-1 Env. HeLa cells were transfected with wild-type proviral DNA or with the indicated mutants, followed by metabolic labeling with [35 S]cysteine. Virions released during the labeling period were pelleted through sucrose, and virion lysates were immunoprecipitated with serum from a patient infected with HIV-1. (B and C) Replication kinetics of MA-deletion mutants in MT4 cells. Target cells were challenged with normalized virus stocks produced by transfected HeLa cells (B) or infected MT4 cells (C). An infectious dose corresponding to 15 ng p24 per 10^6 target cells was used in each experiment. Virus production was monitored by measuring the release of RT activity into the culture supernatants.

the $\Delta 8-87/\Delta CT$ mutant (Figure 1), accelerated virus replication to levels which approached those observed with the parental ΔCT construct (Figure 2B). Even the $\Delta 8-126/\Delta CT$ mutant, which lacks ~90% of MA, replicated with only moderately delayed kinetics (Figure 2B). Moreover, upon further passage of the $\Delta 8-126/\Delta CT$ mutant, the rate of virus production increased to levels similar to those seen with the ΔCT mutant (Figure 2C). The $\Delta 8-87/\Delta CT$ mutant consistently yielded higher peak levels of RT activity than the parental ΔCT construct (Figure 2B). This observation probably reflected differences in cytopathogenicity, because cell numbers dropped more rapidly in cultures infected with the ΔCT virus (data not shown). As expected, the $\Delta 8-87$ and $\Delta 8-126$ mutations prevented virus replication in the presence of wild-type TM protein (data not shown). Metabolic labeling of MT4 cells with [3 H]leucine showed that the parental ΔCT construct and the $\Delta 8-87/\Delta CT$ mutant produced comparable amounts of virus particles at day 5 post-infection (Figure 3D, lanes 2 and 3). Particles released from cells infected with the $\Delta 8-87/\Delta CT$ mutant contained mature CA and NC, but lacked MA. Instead, a product which migrated considerably faster than MA was detectable. This product was only weakly labeled, consistent with the deletion of 13 out of 14 leucine residues from MA (Figure 3D, lane 3).

Efficient replication in the total absence of MA sequences

Since the $\Delta 8-126/\Delta CT$ construct retained the myristylation signal as well as sequences at the C-terminus of MA, we

Fig. 3. Efficient virus assembly and replication in the total absence of MA sequences. (A) Increased viral particle yield upon replacement of MA by a heterologous myristyl anchor. HeLa cells were transfected with proviral DNAs that harbored the ΔCT mutation in *env* and either wild-type or ΔMA mutant *gag*. Lysates of virions released during metabolic labeling with [35 S]cysteine were directly analyzed by SDS-PAGE (left panel). Viral proteins expressed in the transfected cells were immunoprecipitated with patient serum (right panel). The bracket indicates the positions of the C-terminally truncated Env precursor and of mature SU. Pr' indicates the position of the shortened Gag precursor produced by the $\Delta MA/\Delta CT$ mutant. (B) MA is dispensable for the enhancement of particle release by Vpu. HeLa cells were transfected with proviral DNAs that harbor a wild-type or mutated *gag* gene and either an intact or defective *vpu* gene. Lysates of virions released during metabolic labeling with [35 S]cysteine were directly analyzed by SDS-PAGE. (C) Replication kinetics of passaged $\Delta MA/\Delta CT$ virus. Virus recovered several weeks after infection of MT4 cells with HeLa-derived $\Delta MA/\Delta CT$ virus was passaged in fresh MT4 cells and RT activity released into the culture medium was measured. For comparison, the parental ΔCT virus was passaged in parallel. Infections were initiated using an amount of virus equivalent to 15 ng p24 per 10^6 cells. P1, passage 1; P3, passage 3. (D) Protein content of wild-type and mutant HIV-1 virions produced by infected MT4 cells (lanes 1–5). The MT4 cell cultures were infected with virus produced by transfected HeLa cells (lanes 1–3) or with virus previously passaged in MT4 cells (lanes 4 and 5). Lane 6 shows the protein content of HTLV-I virions produced by MT2 cells. Lysates of virions released during metabolic labeling with [3 H]leucine were directly analyzed by SDS-PAGE.

could not exclude that the remaining MA residues were essential for virus replication. To provide a heterologous myristyl anchor, we replaced the N-terminal seven amino acids of MA in the $\Delta 8\text{--}126/\Delta\text{CT}$ construct with the N-terminal nine amino acids of p60^{v-src}, which yielded the $\Delta 1\text{--}126/\Delta\text{CT}$ mutant (Figure 1). The remaining six C-terminal MA residues of the $\Delta 1\text{--}126/\Delta\text{CT}$ mutant were replaced with the six residues that immediately precede protease in the HIV-1 Gag-pol fusion protein. These residues were chosen to retain a processing site at the N-terminus of the CA domain. The resulting $\Delta\text{MA}/\Delta\text{CT}$ provirus has the entire MA domain replaced by a heterologous 15 amino acid sequence (Figure 1).

Transfection into HeLa cells showed that the ΔMA mutation substantially increased viral particle production (Figure 3A and B). Immunoprecipitation from the cell lysates indicated that the increase in particle yield did not result from an increase in the expression levels of the viral structural proteins (Figure 3A). Particles produced by the $\Delta\text{MA}/\Delta\text{CT}$ mutant contained equimolar amounts of CA and NC. Despite the absence of MA, the mutant particles contained significant amounts of SU protein (Figure 3A). Equilibrium centrifugation in sucrose gradients revealed that the lack of MA did not affect particle density (data not shown). It was recently reported that MA is required for the enhancement of virus release by Vpu (Lee *et al.*, 1997). In contrast, we find that the $\Delta 8\text{--}126/\Delta\text{CT}$ and $\Delta\text{MA}/\Delta\text{CT}$ mutants clearly require an intact *vpu* gene for efficient particle production in HeLa cells (Figure 3B).

No evidence for virus replication was initially observed after exposure of MT4 cells to $\Delta 1\text{--}126/\Delta\text{CT}$ or $\Delta\text{MA}/\Delta\text{CT}$ virions produced by transfected HeLa cells. However, in some cultures RT activity began to rise rapidly between 3 and 6 weeks post-infection. Passaged $\Delta\text{MA}/\Delta\text{CT}$ virus reached peak RT levels with only a 3-day delay relative to the ΔCT mutant (Figure 3C). Upon further passage, accelerated replication kinetics were observed which resembled those of the parental ΔCT virus (Figure 3C). Metabolic labeling with [³H]leucine showed that MT4 cells infected with passaged $\Delta\text{MA}/\Delta\text{CT}$ virus released particles which contained CA and p6 as well as RT and IN in similar relative molar amounts as wild-type HIV-1 virions (Figure 3D). NC, which lacks leucine residues, was detected in equimolar amounts relative to CA after metabolic labeling with [³⁵S]cysteine (data not shown). MA was clearly absent from particles produced by the passaged $\Delta\text{MA}/\Delta\text{CT}$ virus (Figure 3D, lane 4). Similar results were obtained with passaged virus derived from MT4 cells infected with the $\Delta 1\text{--}126/\Delta\text{CT}$ mutant (Figure 3D, lane 5). Although MT4 cells harbor HTLV-I proviral DNA, a comparison with the protein profile of virions released by the HTLV-I producer cell line MT2 ruled out the possibility that the MA protein of HTLV-I compensated for that of HIV-1 (Figure 3D, lane 6). This result was anticipated, because HTLV-I Gag protein is only poorly expressed in MT4 cells (Saggiaro *et al.*, 1990).

Replication in other cells

To bypass the host restriction imposed by the truncation of TM (Dubay *et al.*, 1992), MT4 cells infected with the $\Delta 8\text{--}87/\Delta\text{CT}$ or the $\Delta\text{MA}/\Delta\text{CT}$ mutant were co-cultivated with CEM×174 cells. These co-cultures eventually yielded

Fig. 4. Replication of MA-deleted viruses in CEM×174 cells. (A) Replication kinetics of variant isolates of the $\Delta 8\text{--}87/\Delta\text{CT}$ and $\Delta\text{MA}/\Delta\text{CT}$ mutants which could be serially passaged in CEM×174 cells. Infections were initiated with mutant virus that had been passaged repeatedly in CEM×174 cells or with CEM×174-derived wild-type virus. The target cells were exposed to an amount of virus corresponding to 150 ng p24 per 10^6 cells. (B) Analysis of particle- and cell-associated viral proteins. Lysates of virions released from infected CEM×174 cells during labeling with [³⁵S]cysteine were directly analyzed by SDS-PAGE (left panel). Cell-associated viral proteins were immunoprecipitated with patient serum (right panel).

virus which could be serially passaged in CEM×174 cells. Because of the inability of the parental ΔCT virus productively to infect CEM×174 cells (data not shown), replication of the passaged mutant viruses in CEM×174 cells was monitored in comparison with virus harboring intact *gag* and *env* genes. As shown in Figure 4A, the passaged $\Delta 8\text{--}87/\Delta\text{CT}$ and $\Delta\text{MA}/\Delta\text{CT}$ double mutants replicated with delayed kinetics compared with wild-type virus, but produced only ~2-fold lower peak levels of RT activity. CEM×174-derived $\Delta 8\text{--}87/\Delta\text{CT}$ virus also produced high levels of RT activity in Jurkat cells after a 2-week delay relative to wild-type virus, but yielded about one order of magnitude less peak RT activity than wild-type in infected PBMC (data not shown).

PCR amplification of *gag* sequences verified the presence of the original deletions after several passages in CEM×174 cells (data not shown). Metabolic labeling confirmed that CEM×174 or Jurkat cells exposed to passaged MA deletion mutants released particles which contained CA and NC but lacked MA. Of note, the mutants continued to produce a shortened Env precursor, indicating that compensatory changes had occurred which allowed virus replication in CEM×174 or Jurkat cells in the absence of both MA and the cytoplasmic tail of TM (Figure 4B and data not shown).

Electron microscopic analysis of infected cells

To analyze the morphology of particles lacking MA, infected MT4 cells were examined by transmission electron microscopy. Cultures infected with the passaged $\Delta\text{MA}/\Delta\text{CT}$ virus showed numerous, roughly spherical extracellular particles with a central electron-dense core (Figure 5). The particles, which resembled wild-type HIV-1 virions, were covered by a lipid envelope. Occasionally, periodic projections which presumably represented Env glycoprotein spikes were visible on their surface. Appropriate sections showed cone-shaped cores which

Fig. 5. Thin-section electron microscopy of infected MT4 cell cultures. (A and B) Comparison of budding structures and extracellular particles produced in the presence or absence of MA. The arrowheads in (B) point to clearly visible surface projections on a MA-deficient budding structure. (C) Massive budding into intracellular membrane compartments in the absence of the MA core domain. Bars indicate a length of 100 nm.

extended across the entire diameter of the virion, as is typical for HIV-1 (Gelderblom, 1991). However, aberrant core structures appeared more frequently than in wild-type particles. While a few grossly enlarged circular structures with multiple cores were seen, MA-less particles which contained a single core had an average diameter (143 ± 20 nm; $n = 56$) that was comparable with that of particles produced by the parental Δ CT virus (155 ± 16 nm; $n = 44$). In cells infected with the Δ 8-87/ Δ CT, Δ 8-126/ Δ CT or Δ MA/ Δ CT mutants, virus assembly was observed both at the plasma membrane and at intracellular

Fig. 6. Characterization of compensatory mutations which allow efficient HIV-1 replication in MT4 cells in the total absence of MA sequences. (A) Mutations found in the *gag* gene of the replication-competent R0 recombinant. M_{src}, 15 amino acid peptide introduced to provide a heterologous myristyl anchor and processing site (see Figure 1). (B) Replication kinetics of variants of the MA-less mutant Δ MA/ Δ CT which harbor the changes illustrated in (A). MT4 cells were challenged with normalized virus stocks produced by transfected HeLa cells. An amount of virus equivalent to 75 ng per 10^6 target cells was used. Virus replication was monitored by measuring RT activity in the culture supernatants. (C) Effect of compensatory changes in *gag* on CA processing. Lysates of [35 S]methionine-labeled virions produced by transfected HeLa cells were directly analyzed by SDS-PAGE.

membranes (Figure 5), consistent with reports that alterations in MA can cause significant budding into the endoplasmic reticulum and into Golgi elements (Fäcke *et al.*, 1993; Freed *et al.*, 1994). Assembly at intracellular membranes frequently appeared arrested at a late step of budding and did not yield mature particles (Figure 5C).

Isolation of replication-competent proviruses lacking MA coding sequences

After three passages of the Δ MA/ Δ CT virus, HIV-1 *gag* and *pol* sequences were amplified from MT4 cells and used to replace homologous sequences of the Δ CT provirus. A recombinant clone designated R0 yielded virus that replicated with similar kinetics as the parental Δ CT virus (Figure 6B). DNA sequence analysis revealed that the R0 provirus retained the Δ MA mutation, except for a change predicted to result in a Phe→Val substitution at position 13 of the heterologous 15 amino acid sequence (Figure 6A). Other mutations in *gag* changed the codon for Met68 of CA to a codon specifying Val, and the codon for

Arg32 of NC to one specifying Lys. Additionally, the R0 recombinant contained point mutations in *pol*.

To determine which of these mutations were critical for the revertant phenotype, appropriate segments from the R0 recombinant were transferred to the corresponding position of the Δ MA/ Δ CT provirus. The R1 recombinant, which harbored only the Phe→Val change in a Δ MA/ Δ CT background (Figure 6A), did not yield a spreading infection in MT4 cells (Figure 6B). In contrast, the R2 recombinant, which contained the Phe→Val change together with the Met68→Val change in CA (Figure 6A), replicated with only moderately delayed kinetics relative to the R0 recombinant (Figure 6B). Finally, the R3 recombinant, which additionally contained the Arg32→Lys change in NC (Figure 6A), replicated as well as R0 (Figure 6B).

Transfection into HeLa cells showed that Δ MA/ Δ CT virions contained significantly more of a Gag cleavage product which migrated just above CA than virions produced by the R0 and R3 recombinants (Figure 6C). The electrophoretic mobility of this product, which was not present in wild-type virions, indicated that it represented an incompletely processed form of CA with the heterologous myristyl anchor attached to its N-terminus. Virions produced by the R1 recombinant, which harbors only the Phe→Val change, contained even more of this product (Figure 6C). However, virions produced by the R2 recombinant, which additionally harbors the Met68→Val change in CA, contained, almost exclusively, fully processed CA (Figure 6C). Interestingly, an independently obtained revertant also contained the Phe→Val change as well as a Pro147→Thr change in CA. While the Met68→Val mutation mapped to helix IV of the N-terminal core domain whereas the Pro147→Thr mutation affected the linker sequence between the two domains of CA (Gitti *et al.*, 1996; Momany *et al.*, 1996; Gamble *et al.*, 1997), both substitutions had a similar effect on CA processing (data not shown). These results raise the possibility that the changes in CA allowed virus replication, at least in part, by facilitating processing between the heterologous myristyl anchor and CA.

Efficient infection of T cell lines and primary cells in the absence of the globular head of MA

To examine the efficiency of the early phase of virus transmission, we used a single-cycle infection assay in which an *env*-deficient provirus that carries a chloramphenicol acetyltransferase (CAT) gene is complemented by *Env* expressed *in trans* (Helseth *et al.*, 1990). The infectivity of recombinant viruses is assessed by measuring CAT activity in target cells. The single-cycle infection assay revealed that the cytoplasmic domain of TM, although crucial for the productive infection of most target cells (Dubay *et al.*, 1992), is not required for efficient entry or other early steps of the viral life cycle (data not shown). To examine the requirement for MA sequences, variants of pHXBH10 Δ envCAT which carry deletions in MA were constructed. These variants, or the wild-type counterpart, were transfected into HeLa cells together with an expression vector for C-terminally truncated HIV-1_{HXB2} *Env* protein or vesicular stomatitis virus (VSV) G protein. Equivalent amounts of recombinant virus were then used to infect a variety of target cells.

The Δ MA mutation by itself reduced the efficiency of

Fig. 7. Efficiency of the early phase of virus transmission in the absence of MA sequences. The T lymphoid cell lines MT4 (A) and Jurkat (B), or primary human MDM from two different donors (C and D) were exposed to recombinant viruses containing the indicated Gag and Env proteins. Recombinant viruses used in (D) contained either an active or inactive integrase (IN). The results of CAT assays performed on the target cell lysates are shown. All experiments were performed in duplicate.

the early phase of virus replication in MT4 cells >250-fold (Figure 7A). However, virus infectivity was reduced only by ~15-fold relative to wild-type when the Δ MA mutation was combined with compensatory changes in *gag* which allowed virus replication (Figure 7A). The Δ 8–87 mutation, which removed the structurally conserved globular head of MA, had little or no effect on the expression of CAT activity in the human T lymphoid cell lines MT4, C8166, SupT1, CEM-SS and Jurkat (Figure 7A and B and data not shown). In mitogen-stimulated PBMC, Δ 8–87 mutant particles yielded ~4-fold less CAT activity than wild-type particles when complemented with C-terminally truncated HIV-1 *Env*, but retained full infectivity when pseudotyped with VSV G protein (data not shown).

It has been reported that a NLS in the globular head of MA is crucial for HIV-1 replication in non-dividing cells such as MDM, but not in dividing cells (von Schwedler *et al.*, 1994). Since the Δ 8–87 mutation, which removed

the globular head of MA, had relatively little effect on the early phase of virus replication in dividing cells, we also tested its effect in MDM. To this end, we introduced the Δ CT mutation into an expression vector for the Env proteins of the macrophage-tropic HIV-1 isolate ADA. MDM were then exposed to recombinant viruses containing ADA Env. As shown in Figure 7C, the Δ 8–87 mutation reduced the efficiency of infection of MDM ~15-fold. As expected, hardly any CAT activity was detectable following exposure of MDM to recombinant viruses containing T cell-adapted rather than macrophage-tropic Env (Figure 7C). Remarkably, pseudotyping with VSV G protein completely suppressed the defect caused by the Δ 8–87 mutation and allowed efficient infection of MDM in the absence of the globular head of MA (Figure 7C). This finding was reproducible with MDM from four different donors (Figure 7D and data not shown). The observed CAT activity was reduced to background levels when infections were carried out in the presence of 5 μ M azidothymidine (data not shown). Furthermore, as shown in Figure 7D, the transfer of CAT activity to MDM was blocked by a single amino acid substitution in IN (Asp116→Ala) which is known to abolish IN activity (Wiskerchen and Muesing, 1995). These results indicate that the entire globular head of MA is dispensable for reverse transcription, nuclear import and integration in MDM.

Discussion

This study demonstrates that MA, one of the major structural proteins of mature HIV-1 virions, is not absolutely required for efficient virus replication. While MA-deleted viruses were unable to incorporate the wild-type HIV-1 Env protein complex, this defect could be corrected by a second-site mutation, which removed the large cytoplasmic domain of TM. Moreover, the second-site mutation allowed efficient HIV-1 replication in the absence of MA sequences. Interestingly, mutants with large deletions in MA proved to be considerably more viable than those with smaller deletions. Virus replication was particularly efficient in the absence of the entire globular core of MA. Remarkably, even a mutant which lacked all of MA but rather contained a heterologous myristyl anchor was able to replicate efficiently after the acquisition of compensatory changes in *gag*.

One previous study showed that the second half of the MA protein of the oncoretrovirus RSV is dispensable for viral infectivity (Nelle and Wills, 1996). However, even small alterations in the N-terminal half of RSV MA completely abolished virus budding or infectivity (Nelle and Wills, 1996; Parent *et al.*, 1996). In the case of HIV-1, relatively subtle alterations in MA often had drastic effects on HIV-1 morphogenesis and infectivity. It has been reported that amino acid substitutions in the globular core of MA which are unlikely to affect the N-terminal attachment of myristic acid can significantly reduce or even block HIV-1 particle assembly (Yuan *et al.*, 1993; Freed *et al.*, 1994; Morikawa *et al.*, 1995; Cannon *et al.*, 1997). Also, relatively small alterations throughout MA which allowed particle formation often prevented virus replication (Yu *et al.*, 1992b; Dorfman *et al.*, 1994; Cannon *et al.*, 1997). While the globular domain of MA is

known to be critical for the accommodation of the long cytoplasmic tail of HIV-1 TM (Freed and Martin, 1995; Mammano *et al.*, 1995), certain deletions near the C-terminus of MA which allowed Env protein incorporation nevertheless severely impaired virus infectivity (Yu *et al.*, 1992a). A large deletion which essentially removed the globular core of MA was found to redirect particle assembly from the plasma membrane to intracellular locations, with a concomitant decrease in the release of extracellular particles (Fäcke *et al.*, 1993). In contrast, others observed efficient formation of extracellular particles when $\geq 80\%$ of MA was deleted (Wang *et al.*, 1993; Lee and Linial, 1994). While one of these studies also showed that the globular core of MA is not absolutely required for early steps of the HIV-1 replication cycle (Wang *et al.*, 1993), ours is the first to demonstrate that MA-deleted virions can initiate a spreading infection.

Since the cytoplasmic tail of TM, although crucial for replication in most cells (Dubay *et al.*, 1992), was not required for efficient entry, we were able to assess the infectivity of MA-deleted virions for a variety of target cells. In the cell lines tested, the early phase of virus replication was not, or was only moderately, affected by the absence of the globular head, the portion of MA which is structurally conserved among widely divergent retroviruses (Christensen *et al.*, 1996; Conte *et al.*, 1997). The ability of the mutant particles to infect primary human target cells from different donors varied somewhat, but was reduced by no more than 4-fold for PBMC and 15-fold for MDM. Interestingly, no defect in infectivity for primary target cells was apparent when the mutant particles were complemented with VSV G protein. This effect of VSV G was not due to a general enhancement of virion infectivity, because particles formed by wild-type Gag were at least as infectious for primary cells when complemented with HIV-1 Env rather than VSV G. While HIV-1 Env promotes direct fusion of the viral envelope with the plasma membrane (Stein *et al.*, 1987), internalization of HIV-1 particles pseudotyped with VSV G occurs via receptor-mediated endocytosis (Aiken, 1997). Thus, MA may help to overcome a barrier to viral entry through the plasma membrane which can be circumvented by changing the route of entry.

It has been reported that the globular head of MA contains a basic-type NLS which is critical for the import of the viral preintegration complex into the nucleus of non-dividing cells such as MDM (Bukrinsky *et al.*, 1993; Heinzinger *et al.*, 1994). Disruption of the MA NLS had no effect on HIV-1 replication in proliferating MT4 cells but impaired replication in growth-arrested cells and in MDM, particularly in the absence of Vpr, a second nucleophilic virion component (Bukrinsky *et al.*, 1993; Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994). However, more recent studies failed to provide supporting evidence for a role of the N-proximal basic region of MA in post-entry nuclear import (Freed *et al.*, 1995; Fouchier *et al.*, 1997). In the present study, HIV-1 particles which lacked both Vpr and the globular head of MA, when pseudotyped with VSV G, were as infectious for MDM as particles that lacked only Vpr. Since the transfer of CAT activity to MDM was absolutely dependent on the presence of a functional IN, our findings imply that the

removal of the globular head of MA did not affect post-entry events up to integration.

Intriguingly, mutants with large deletions in MA exhibited an increased capacity to form viral particles. A particularly pronounced increase in particle yield was observed when the entire MA domain was deleted and replaced by the myristylated N-terminus of the oncoprotein p60^{src}. However, significant increases were also seen when the autologous myristylation signal was retained. Of note, the mutants lacked the N-proximal basic region in MA which has been implicated in Gag membrane binding (Zhou *et al.*, 1994). While our results do not exclude the possibility that the basic region confers specificity to the interaction of Gag with cellular membranes, they demonstrate that this region is dispensable for the stable membrane association of Gag.

Our finding that large deletions in MA increased particle yields may be explained by a recently proposed myristyl switch model of Gag membrane binding (Zhou and Resh, 1996). According to this model, the N-terminal myristate can be sequestered by MA and would be available for membrane binding only after a conformational change. Our results are consistent with the possibility that Gag membrane binding and, as a consequence, particle production are increased in the absence of the globular domain of MA because the myristyl moiety can no longer be sequestered and is therefore constitutively exposed. A myristyl switch mechanism could conceivably have evolved to ensure the selective targeting of Gag to the plasma membrane. One would then expect that constitutive exposure of the myristyl group should lead to non-selective membrane binding, which may explain the tendency of MA deletion mutants to bud into intracellular membrane compartments.

HIV-1 MA has been shown to interact with the cytoplasmic domain of TM *in vitro*, and it has been proposed that this interaction directs the Env complex into assembling virions (Cosson, 1996). However, HIV-1 particles readily incorporate heterologous, apparently unrelated Env glycoproteins with much shorter cytoplasmic domains (Hunter, 1994), indicating that a specific interaction with MA is not always required. The present study supports this view by demonstrating that efficient incorporation of the HIV-1 Env protein complex can be achieved in the absence of both MA and the cytoplasmic domain of TM. The function of the unusually long cytoplasmic domain of TM remains unclear, particularly in view of our finding that this domain is not required for efficient entry into a variety of target cells.

Our results demonstrate that MA does not belong to those virion constituents which are indispensable for the completion of the unique retroviral replication cycle. MA-deleted virions in essence represent fusion-competent lipid vesicles which contain a viral core. The remarkable replication potential of such structures exhibited in the present study raises the possibility that the cores by themselves may be fully capable of initiating the retroviral replication cycle, provided they can be purified intact and introduced into a permissive cellular environment.

Materials and methods

Plasmids

HXBH10, the parental HIV-1 proviral clone used, encodes a C-terminally truncated Vpr protein which was reported to be unable to substitute for

the nuclear import function of MA in MDM (von Schwedler *et al.*, 1994). HXBH10ΔCT, a variant of HXBH10 with a premature termination codon in place of codon 713 of *env*, and HXBH10ΔenvCAT, a derivative with a deletion in *env* and a CAT gene in place of *nef*, have been described (Mammano *et al.*, 1995). In-frame deletions in the MA coding region were generated by recombination of previously described MA mutants (Dorfman *et al.*, 1994). In each case, two foreign codons specifying Ser-Arg were inserted in place of the deleted MA codons. To generate the Δ1–126 mutation, the first nine codons of the HIV-1 *gag* gene were replaced by those for Rous sarcoma virus *src*, followed by codons specifying Ser-Arg to create a *XbaI* site. This was achieved by site-directed mutagenesis using a 74mer (sense orientation: 5'-GGAGGCTAGAAGGAGAGAGATGGGTAGCAGCAAGAGCAAGCCTAAGTCTAGAGGGGAGAATTAGATCGATGGG-3'). The *XbaI* site was then used for recombination with the *XbaI* site generated by the previously described N126→R mutation in MA (Dorfman *et al.*, 1994). The ΔMA mutation, which was predicted to substitute the sequence Met-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Thr-Val-Ser-Phe-Asn-Phe for MA, was generated by site-directed mutagenesis using an 88mer (sense orientation: 5'-GGAGGCTAGAAGGAGAGAGATGGGTAGCAGCAAGAGCAAGCCTAAGACAGTAAGCTTTAATTTCCCTATAGTGCAACATCCAGGGG-3').

To generate *vpu* variants of MA mutants, a *SalI*–*NheI* fragment (nt 5789–7263) was replaced by the corresponding fragment from HXB2, which harbors a defective *vpu* gene. The Asp116→Ala mutation in the IN coding region was generated by site-directed mutagenesis using the oligonucleotide 5'-CAATACATACCGCGAATGGCAGC-3'. The mutation was subcloned into HXBH10ΔenvCAT on a *Pf*MI fragment (nt 3491–5302).

The pEnv_{HXB} plasmid, an expressor for HIV-1_{HXB2} Env, was generated by inserting a *SalI*–*XhoI* fragment from pSVIIIenv (Helseth *et al.*, 1990) into the expression vector pBJ5stop (obtained from Richard Bram). The pEnv_{HXB}ΔCT derivative, which expresses a truncated HIV-1_{HXB2} Env that lacks the 144 C-terminal amino acids of the cytoplasmic domain of TM, was obtained by replacing a *KpnI*–*XhoI* fragment (nt 6347–8900) in pEnv_{HXB} by the corresponding fragment from HXBH10ΔCT. The pEnv_{ADA}ΔCT construct, an expressor plasmid for C-terminally truncated HIV-1_{ADA} Env, was created by replacing a *KpnI*–*BsmI* fragment (nt 6347–8054) in pEnv_{HXB}ΔCT by an equivalent fragment from the macrophage-tropic isolate ADA. The pHCMV-G plasmid (Yee *et al.*, 1994) expressing the VSV G protein was obtained from Theodore Friedmann.

Cells

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). The T lymphoid cell lines MT4, MT2, C8166, SupT1, CEM-SS and Jurkat clone E6-1, and the T-B cell hybrid CEM×174, were maintained in RPMI 1640 medium with 10% FCS. Human PBMC were isolated from the blood of normal donors by Ficoll-Hypaque density gradient centrifugation. Monocytes were purified from PBMC by adherence to plastic. The purification procedure typically yielded >95% CD14⁺ cells as assayed by flow cytometry. MDM were obtained by culturing purified monocytes for 2 weeks in RPMI 1640 and 10% FCS without additional cytokines. PBMC were stimulated for 24 h with phytohemagglutinin (1 μg/ml) and maintained in RPMI 1640 containing 10% FCS and 10% of a human interleukin-2 preparation (Hemagen Diagnostics, Columbia, MD).

Transfections and infections

HeLa cells were transfected by a calcium phosphate precipitation technique. Viral stocks were passed through 0.45 μm pore size filters, and equivalent amounts of ³²P-RT activity measured as described (Willey *et al.*, 1988) were used to inoculate 3×10⁶ target cells in 2 ml culture medium. The amount of p24^{gag} in viral stocks was measured using a commercial ELISA (Du Pont). Following overnight incubation, the target cells were washed and resuspended in 5 ml culture medium. Viral replication was monitored by measuring ³²P-RT activity in the culture supernatants.

Viral protein analysis

Transfected or infected cells were metabolically labeled for 12 h with [³⁵S]cysteine, [³⁵S]methionine or [³H]leucine (50 μCi/ml). Labeled cells were lysed in RIPA buffer (140 mM NaCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS), and viral proteins were immunoprecipitated with serum from an individual infected with HIV-1 and subjected to SDS-PAGE. Viral particles released during the labeling period were pelleted through sucrose cushions (in PBS) for 90 min at 4°C and 26 000 r.p.m. in a Beckman

SW 28 rotor. Pelleted virions were lysed in RIPA buffer and viral proteins were either directly analyzed by SDS-PAGE or immunoprecipitated prior to electrophoresis.

Molecular cloning of revertant sequences

Total DNA was purified from MT4 cells with the QIAamp Blood Kit (Quiagen, Chatsworth, CA) 5 days post-infection. Two contiguous proviral segments containing *gag* and *pol* sequences were amplified with primer pairs flanking unique restriction sites. The R0 recombinant was obtained by inserting a *Bss*HII-*Bcl*I fragment (nt 710–2432) of segment 1 together with a *Bcl*I-*Sal*I fragment (nt 2432–5789) of segment 2 into the Δ CT proviral construct.

Env-complementation assay

To produce recombinant virions, HeLa cells were cotransfected with wild-type or mutant HXBH10 Δ envCAT and an expression plasmid for HIV-1 Env, VSV G protein or empty vector. Equivalent ³²P-RT units of filtered supernatants (corresponding to ~25 ng p24^{gag}) were used to inoculate 2×10⁶ target cells in 6-well dishes. The cells were incubated for 3–5 days, lysed, and assayed for CAT activity as described previously (Helseth *et al.*, 1990). To facilitate a comparison of relative infectivities, lysate from cells infected with recombinant virus with an intact MA protein was serially 2-fold diluted with lysate from uninfected cells.

Electron microscopy

Five days post-infection, MT4 cells were fixed in 2.5% glutaraldehyde for 30 min, sedimented at 200 g, and overlaid with 2.5% glutaraldehyde in PBS. Cell pellets were post-fixed in 1% osmium tetroxide, embedded in agar, treated with 2% uranyl acetate, and finally embedded into Epon. Ultrathin sections were poststained with lead citrate and examined in a Zeiss 10 A transmission electron microscope at 60 kV. Measurements of virus dimensions were taken from the negatives of electron micrographs. Particles were measured only when the full length of the mature core was apparent. Viral particle size was evaluated by calculating the mean of the minimum and maximum diameters for each virion.

Acknowledgements

We thank Drs Fabrizio Mammano, Theodore Friedmann and Richard Bram for gifts of plasmids. This work was supported by National Institutes of Health grants AI29873, AI28691 (Center for AIDS Research) and CA06516 (Cancer Center), and by a gift from the G.Harold and Leila Y.Mathers Charitable Foundation.

References

- Aiken, C. (1997) Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. *J. Virol.*, **71**, 5871–5877.
- Bryant, M. and Ratner, L. (1990) Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl Acad. Sci. USA*, **87**, 523–527.
- Bukrinskaya, A.G., Ghorpade, A., Heinzinger, N.K., Smithgall, T.E., Lewis, R.E. and Stevenson, M. (1996) Phosphorylation-dependent human immunodeficiency virus type 1 infection and nuclear targeting of viral DNA. *Proc. Natl Acad. Sci. USA*, **93**, 367–371.
- Bukrinsky, M.I., Sharova, N., Dempsey, M.P., Stanwick, T.L., Bukrinskaya, A.G., Haggerty, S. and Stevenson, M. (1992) Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc. Natl Acad. Sci. USA*, **89**, 6580–6584.
- Bukrinsky, M.I. *et al.* (1993) A nuclear localization signal within HIV-1 matrix protein that governs infection of non-diving cells. *Nature*, **365**, 666–669.
- Cannon, P.M., Matthews, S., Clark, N., Byles, E.D., Iourin, O., Hockley, D.J., Kingsman, S.M. and Kingsman, A.J. (1997) Structure–function studies of the human immunodeficiency virus type 1 matrix protein, p17. *J. Virol.*, **71**, 3474–3483.
- Christensen, A.M., Massiah, M.A., Turner, B.G., Sundquist, W.I. and Summers, M.F. (1996) Three-dimensional structure of the HTLV-II matrix protein and comparative analysis of matrix proteins from the different classes of pathogenic human retroviruses. *J. Mol. Biol.*, **264**, 1117–1131.
- Conte, M.R., Klikova, M., Hunter, E., Ruml, T. and Matthews, S. (1997) The three-dimensional solution structure of the matrix protein from the type D retrovirus, the Mason-Pfizer monkey virus, and implications for the morphology of retroviral assembly. *EMBO J.*, **16**, 5819–5826.
- Cosson, P. (1996) Direct interaction between the envelope and matrix proteins of HIV-1. *EMBO J.*, **15**, 5783–5788.
- Dorfman, T., Mammano, F., Haseltine, W.A. and Göttinger, H.G. (1994) Role of the matrix protein in the virion association of the human immunodeficiency virus type 1 envelope glycoprotein. *J. Virol.*, **68**, 1689–1696.
- Dubay, J.W., Roberts, S.J., Hahn, B.H. and Hunter, E. (1992) Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. *J. Virol.*, **66**, 6616–6625.
- Fäcke, M., Janetzko, A., Shoeman, R.L. and Kräusslich, H.-G. (1993) A large deletion in the matrix domain of the human immunodeficiency virus *gag* gene redirects virus particle assembly from the plasma membrane to the endoplasmic reticulum. *J. Virol.*, **67**, 4972–4980.
- Fouchier, R.A.M., Meyer, B.E., Simon, J.H.M., Fischer, U. and Malim, M.H. (1997) 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. *EMBO J.*, **16**, 4531–4539.
- Freed, E.O. and Martin, M.A. (1995) Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix. *J. Virol.*, **69**, 1984–1989.
- Freed, E.O., Orenstein, J.M., Buckler-White, A.J. and Martin, M.A. (1994) Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. *J. Virol.*, **68**, 5311–5320.
- Freed, E.O., Englund, G. and Martin, M.A. (1995) Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection. *J. Virol.*, **69**, 3949–3954.
- Freed, E.O., Englund, G., Maldarelli, F. and Martin, M.A. (1997) Phosphorylation of residue 131 of HIV-1 matrix is not required for macrophage infection. *Cell*, **88**, 171–174.
- Gallay, P., Swingle, S., Aiken, C. and Trono, D. (1995a) HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *Cell*, **80**, 379–388.
- Gallay, P., Swingle, S., Song, J., Bushman, F. and Trono, D. (1995b) HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell*, **83**, 569–576.
- Gallay, P., Hope, T., Chin, D. and Trono, D. (1997) HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl Acad. Sci. USA*, **94**, 9825–9830.
- Gamble, T.R., Yoo, S., Vajdos, F.F., von Schwedler, U., Worthylake, D.K., Wang, H., McCutcheon, J.P., Sundquist, W.I. and Hill, C.P. (1997) Structure of the carboxy-terminal dimerization domain of the HIV-1 capsid protein. *Science*, **278**, 849–853.
- Gelderblom, H.R. (1991) Assembly and morphology of HIV: potential effect of structure on viral function. *AIDS*, **5**, 617–638.
- Gitti, R.K., Lee, B.M., Walker, J., Summers, M.F., Yoo, S. and Sundquist, W.I. (1996) Structure of the amino-terminal core domain of the HIV-1 capsid protein. *Science*, **273**, 231–235.
- Göttinger, H.G., Sodroski, J.G. and Haseltine, W.A. (1989) Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc. Natl Acad. Sci. USA*, **86**, 5781–5785.
- Heinzinger, N.K. *et al.* (1994) The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc. Natl Acad. Sci. USA*, **91**, 7311–7315.
- Helseth, E., Kowalski, M., Gabuzda, D., Olshevsky, U., Haseltine, W. and Sodroski, J. (1990) Rapid complementation assay measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. *J. Virol.*, **64**, 2416–2420.
- Hill, C.P., Worthylake, D., Bancroft, D.P., Christensen, A.M. and Sundquist, W.I. (1996) Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. *Proc. Natl Acad. Sci. USA*, **93**, 3099–3104.
- Hunter, E. (1994) Macromolecular interactions in the assembly of HIV and other retroviruses. *Semin. Virol.*, **5**, 71–83.
- Kräusslich, H.-G. and Welker, R. (1996) Intracellular transport of retroviral capsid components. *Curr. Top. Microbiol. Immunol.*, **214**, 25–63.

- Lee,P.P. and Linial,M.L. (1994) Efficient particle formation can occur if the matrix domain of human immunodeficiency virus type 1 Gag is substituted by a myristylation signal. *J. Virol.*, **68**, 6644–6654.
- Lee,Y.-H., Schwartz,M.D. and Panganiban,A.T. (1997) The HIV-1 matrix domain of Gag is required for Vpu responsiveness during particle release. *Virology*, **237**, 46–55.
- Lewis,P., Hensel,M. and Emerman,M. (1992) Human immunodeficiency virus infection of cells arrested in the cell cycle. *EMBO J.*, **8**, 3053–3058.
- Mammano,F., Kondo,E., Sodroski,J., Bukovsky,A. and Göttinger,H.G. (1995) Rescue of human immunodeficiency virus type 1 matrix protein mutants by envelope glycoproteins with short cytoplasmic domains. *J. Virol.*, **69**, 3824–3830.
- Massiah,M.A., Starich,M.R., Paschall,C., Summers,M.F., Christensen,A.M. and Sundquist,W.I. (1994) Three-dimensional structure of the human immunodeficiency virus type 1 matrix protein. *J. Mol. Biol.*, **244**, 198–223.
- Matthews,S. *et al.* (1994) Structural similarity between the p17 matrix protein of HIV-1 and interferon- α . *Nature*, **370**, 666–668.
- Momany,C. *et al.* (1996) Crystal structure of dimeric HIV-1 capsid protein. *Nature Struct. Biol.*, **3**, 763–770.
- Morikawa,Y., Kishi,T., Zhang,W.H., Nermut,M.V., Hockley,D.J. and Jones,I.M. (1995) A molecular determinant of human immunodeficiency virus particle assembly located in matrix antigen p17. *J. Virol.*, **69**, 4519–4523.
- Nelle,T.D. and Wills,J.W. (1996) A large region within the Rous sarcoma virus matrix protein is dispensable for budding and infectivity. *J. Virol.*, **70**, 2269–2276.
- Parent,L.J., Wilson,C.B., Resh,M.D. and Wills,J.W. (1996) Evidence for a second function of the MA sequence in the Rous sarcoma virus Gag protein. *J. Virol.*, **70**, 1016–1026.
- Saggiaro,D., Panozzo,M. and Chieco-Bianchi,L. (1990) Human T-lymphotropic virus type I transcriptional regulation by methylation. *Cancer Res.*, **50**, 4968–4973.
- Stein,B.S., Gowda,S.D., Lifson,J.D., Penhallow,R.C., Bensch,K.G. and Engleman,E.G. (1987) pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell*, **49**, 659–668.
- von Schwedler,U., Kornbluth,R.S. and Trono,D. (1994) The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. *Proc. Natl Acad. Sci. USA*, **91**, 6992–6996.
- Wang,C.-T., Zhang,Y., McDermott,J. and Barklis,E. (1993) Conditional infectivity of a human immunodeficiency virus matrix domain deletion mutant. *J. Virol.*, **67**, 7067–7076.
- Weinberg,J.B., Matthews,T.J., Cullen,B.R. and Malim,M.H. (1991) Productive human immunodeficiency virus type 1 (HIV-1) infection of nonproliferating human cells. *J. Exp. Med.*, **174**, 1477–1482.
- Wilk,T., Pfeiffer,T. and Bosch,V. (1992) Retained *in vitro* infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the *env* gene product. *Virology*, **189**, 167–177.
- Willey,R.L., Smith,D.H., Lasky,L.A., Theodore,T.S., Earl,P.L., Moss,B., Capon,D.J. and Martin,M.A. (1988) *In vitro* mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. *J. Virol.*, **62**, 139–147.
- Wiskerchen,M. and Muesing,M.A. (1995) Human immunodeficiency virus type 1 integrase: effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells. *J. Virol.*, **69**, 376–386.
- Yee,J.-K., Friedmann,T. and Burns,J.C. (1994) Generation of high-titer pseudotyped retroviral vectors with very broad host range. *Methods Cell Biol.*, **43**, 99–112.
- Yu,X., Yu,Q.-C., Lee,T.-H. and Essex,M. (1992a) The C terminus of human immunodeficiency virus type 1 matrix protein is involved in early steps of the virus life cycle. *J. Virol.*, **66**, 5667–5670.
- Yu,X., Yuan,X., Matsuda,Z., Lee,T.-H. and Essex,M. (1992b) The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. *J. Virol.*, **66**, 4966–4971.
- Yuan,X., Yu,X., Lee,T.-H. and Essex,M. (1993) Mutations in the N-terminal region of human immunodeficiency virus type 1 matrix protein block intracellular transport of the Gag precursor. *J. Virol.*, **67**, 6387–6394.
- Zhou,W. and Resh,M.D. (1996) Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. *J. Virol.*, **70**, 8540–8548.
- Zhou,W., Parent,L.J., Wills,J.W. and Resh,M.D. (1994) Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J. Virol.*, **68**, 2556–2569.

Received January 23, 1998; revised and accepted March 6, 1998