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Role of the Basic Domain of Human Immunodeficiency Virus Type 1 Matrix in Macrophage Infection

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The matrix domain of the human immunodeficiency virus type 1 (HIV-1) Gag protein contains a highly basic region near its amino terminus. It has been proposed that this basic domain, in conjunction with the HIV-1 accessory protein Vpr, is responsible for the localization of the HIV-1 preintegration complex to the nucleus in nondividing cells. It has also been postulated that the matrix basic domain assists in the targeting of the HIV-1 Gag precursor $Pr55^{Gag}$ to the plasma membrane during virus assembly. To evaluate the role of this highly basic sequence during infection of primary human monocyte-derived macrophages, single- and double-amino-acid-substitution mutations were introduced, and the effects on virus particle production, Gag protein processing, envelope glycoprotein incorporation into virus particles, and virus infectivity in the CEM(12D-7) T-cell line, peripheral blood mononuclear cells, and primary human monocyte-derived macrophages were analyzed. Although modest effects on virus particle production were observed with some of the mutants, none abolished infectivity in primary human monocyte-derived macrophages. In contrast with previously reported studies involving some of the same matrix basic domain mutants, infectivity in monocyte-derived macrophages was retained even when combined with a *vpr* mutation.

The Gag proteins of human immunodeficiency virus type 1 (HIV-1) are synthesized as a polyprotein precursor, $Pr55^{Gag}$, which is proteolytically processed to generate the mature p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), and $p6^{Gag}$ proteins (28). In virus particles, the MA domain of Gag is localized to the inner face of the lipid bilayer of the viral envelope, to which it is attached by a myristic acid moiety (for reviews, see references 19 and 20). A number of functions have been proposed for the HIV-1 MA. These include roles in the targeting of Gag to the plasma membrane (4, 21, 33, 51, 52), virus particle assembly and release (4, 6, 17, 21, 44), envelope glycoprotein incorporation into virus particles (10, 15, 50), virus entry (16, 49), and localization of the virus preintegration complex to the nucleus of infected, nondividing cells (5, 22, 43).

Lentiviral MA proteins contain a highly basic domain near their amino termini (32). In the case of HIV-1, this basic region is located between MA amino acid residues 17 and 31. Two primary roles have been postulated for this basic domain: (i) the association of $Pr55^{Gag}$ with the plasma membrane during virus assembly (51, 52) and (ii) translocation of the viral preintegration complex to the nucleus after entry (5, 22, 43).

Lentiviruses, including HIV-1, are able to infect nondividing cells, while oncoretroviruses require that the host cell pass through mitosis for a productive infection to become established (3, 23, 29, 38, 42). The preintegration complex of lentiviruses must therefore have the ability to target and enter the nucleus of a nondividing host cell. Bukrinsky and colleagues reported (5) that mutation of the two adjacent Lys residues at amino acids 25 and 26 (within the highly basic domain of MA) blocked infection of nondividing cells. Although we subsequently reported that mutation of these residues did not block infection of primary human monocyte-derived macrophages (MDMs) (13), the possibility remained that mutation of other basic residues in this region of MA might block infectivity for primary MDMs. In particular, if the basic domain of MA were a member of the bipartite class of nuclear localization signals (9, 36), mutations at the N and C termini might have a greater effect on macrophage infection than mutations in the center of this basic region.

After the publication of the study by Bukrinsky et al. (5), the same investigators reported that the virus isolate used in their earlier studies contained a defective *vpr* gene and that the block to infection of nondividing cells, originally interpreted as being caused by MA basic region mutations, was observed only in the context of a Vpr-defective virus (22). von Schwedler et al. also recently reported a loss of infectivity in human MDMs for HIV-1 mutants containing both MA basic domain and *vpr* mutations (43).

To further evaluate the role of the highly basic region of HIV-1 MA in the virus life cycle, particularly with respect to macrophage infection, we introduced a series of single- and double-amino-acid-substitution mutations affecting the basic residues throughout this domain, including some previously reported to impair infectivity in MDMs (22, 43). The mutations were analyzed for effects on Gag expression and processing, virus particle production, incorporation of envelope glycoproteins into virus particles, and virus infectivity in a T-cell line, peripheral blood mononuclear cells, and primary human MDMs. Two of the mutants were also analyzed for replication in MDMs in the context of a *vpr* mutation. Our results indicate that single- or double-amino-acid substitution of basic residues in the MA basic domain, either alone or in combination with a *vpr* mutation, reduces but does not abolish the ability of HIV-1 to productively infect cultures of primary human MDMs.

Effect of MA mutations on virus particle production. We recently demonstrated that single-amino-acid mutations in HIV-1 MA can block virus particle production (17) and envelope glycoprotein incorporation into virus particles (15). To assess the role(s) of the MA basic region in MA function, we introduced a series of single- and double-amino-acid-substitution mutations between MA amino acids 17 and 31 (Fig. 1) in the full-length HIV-1 molecular clone pNL4-3 (1) as described

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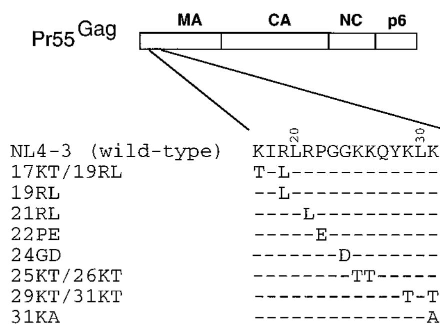

FIG. 1. Mutagenesis of the basic domain of MA. The wild-type NL4-3 sequence is indicated in the single-letter amino acid code. The single- or double-amino-acid substitutions are shown, with the name of the mutation listed below NL4-3. Dashes indicate amino acid sequence identity with NL4-3.

previously (17). Preliminary characterization of several of these mutants was reported previously (17). To analyze the effects of the MA mutations on virus particle production, HeLa cells were transfected in parallel with wild-type or mutant pNL4-3 clones, and the amount of particle-associated reverse transcriptase (RT) activity released into the transfected cell medium was quantified (17). The results, presented in Table 1, indicate that two of the basic region mutations (25KT/26KT and 29KT/31KT) modestly and consistently reduce virus particle production (two- to threefold) compared with that of the wild type. Other mutations in this domain have no significant effect on the production of virus particle-associated RT activity.

Mutant Gag expression and processing and envelope incorporation into virus particles. To examine the expression and processing of the MA mutants and to analyze further the effects of the mutations on the release of virion-associated proteins, we transfected HeLa cells in parallel with wild-type or mutant proviral clones, metabolically labeled the transfected cells with [^{35}S]Cys, and radioimmunoprecipitated the cell- and virion-associated proteins as previously described (14, 47). The results of this analysis (Fig. 2) indicate that none of the mutations block envelope glycoprotein incorporation into virus particles, as evidenced by the presence of the surface envelope glycoprotein gp120 in all virus preparations. Several of the mutations, however (i.e., 25KT/26KT, 31KA, and 29KT/31KT), do cause a modest reduction in the amount of gp120, relative to p24, incorporated into virus particles. As noted above, these mutations also reduce virus particle production. A reduction in the levels of virion gp120 resulting from amino acid 29 and 31 mutations was not unanticipated, because we recently demonstrated that a single-amino-acid substitution at residue 30 (Leu→Glu) abolished HIV-1 envelope glycoprotein incorporation into virus particles (15).

TABLE 1. Effects of MA basic domain mutations on virus particle production

Mutant	Mean virion-associated RT activity ± SD[a]
pNL4-3 (wild type)	100
17KT/19RL	120 ± 22
19RL	91 ± 26
21RL	148 ± 36
22PE	130 ± 18
24GD	128 ± 15
25KT/26KT	47 ± 8
29KT/31KT	34 ± 13
31KA	68 ± 6

[a] Relative virus particle-associated RT activity released into the medium of transfected HeLa cells. Data represent the averages of at least three independent transfection assays. Some of the data in this table were presented previously (17) and are recapitulated here for comparison with data for the new mutants.

Replication of MA mutants in the CEM(12D-7) T-cell line. We were next interested in analyzing the effects of the MA mutations on the establishment of a productive infection in T cells. The CEM(12D-7) cell line (37) was transfected in parallel with wild-type pNL4-3 or pNL4-3 MA mutants. The production of RT activity was assayed over time to monitor virus replication in the transfected cultures (Fig. 3). Although all mutants were capable of establishing a productive infection in CEM(12D-7) cells, several, including the 25KT/26KT mutant, displayed a consistent but brief delay until peak RT activity or produced somewhat less virus at peak RT activity relative to the wild type.

Replication of MA mutants in MDMs. Nondividing, fully differentiated cells of the monocyte/macrophage lineage are thought to be an important target for HIV-1 infection in vivo (18, 25, 30, 31, 40). Because it was reported that the highly basic domain of MA is required for infection of nondividing cells (5), we evaluated the effect of MA basic region mutations on infection of primary human MDMs. We constructed a macrophage-tropic derivative of pNL4-3 by removing the 1.7-kbp fragment between the *Kpn*I site in the gp120 coding region and the *Bsm*I site in gp41 from the macrophage-tropic clone pAD8-1 (41) and substituting this fragment for the corresponding region of pNL4-3 (pNL4-3 *env* nucleotides 104 to 1820 [32]). The resulting clone, pNL(AD8), efficiently infects primary human MDMs, unlike the parent, pNL4-3, which is unable to productively infect this cell type (13). MA mutations were introduced into pNL(AD8) by substituting the *Bss*HII-*Sph*I fragment (pNL4-3 nucleotides 711 to 1442 [32]) containing the mutations from the previously constructed mutant pNL4-3 clones for the corresponding sites in pNL(AD8). HeLa cells were transfected (14) in parallel with wild-type or MA mutant proviral clones to generate virus pools for macrophage infections.

Cultures of fully differentiated MDMs were prepared by the method of Lazdins et al. (26, 27). Elutriated monocytes were allowed to differentiate for 14 days in bacteriological petri plates (Sterilin 109 or Corning S27050-100) in Dulbecco's modified Eagle's medium supplemented with 10% fresh human serum, 2 mM L-glutamic acid, 1 mM sodium pyruvate, 25 U of penicillin per ml, and 25 μg of streptomycin per ml. After the 14-day preculture differentiation period, cells were transferred to 96-well Nunc tissue culture plates (1-67008) at 10^5 cells per well. A recent report suggested that cultures of primary human macrophages contain a small proportion of actively proliferating cells (39). In that report, however, the cells were allowed to differentiate for only 5 days, instead of 14 days as in the present study. The nonproliferating status of MDMs prepared with the 14-day preculture incubation has been previously determined by [^{3}H]thymidine incorporation analyses (12). The MDM cultures were infected with equivalent amounts of virus (normalized for RT activity) 1 day after plating in the 96-well plates. Virus (10^5 RT cpm per well) was adsorbed at 37°C for 1 h in 50 μl of macrophage growth medium (described above), after which time 200 μl of macrophage growth medium was added per well. Culture medium was replaced with fresh medium every 2 days; 150-μl aliquots were reserved at each time point for RT assays, which were performed essentially as described previously (48) with the addition of 0.8 mM EDTA to the RT cocktail.

The virus replication kinetics in MDM cultures are pre-

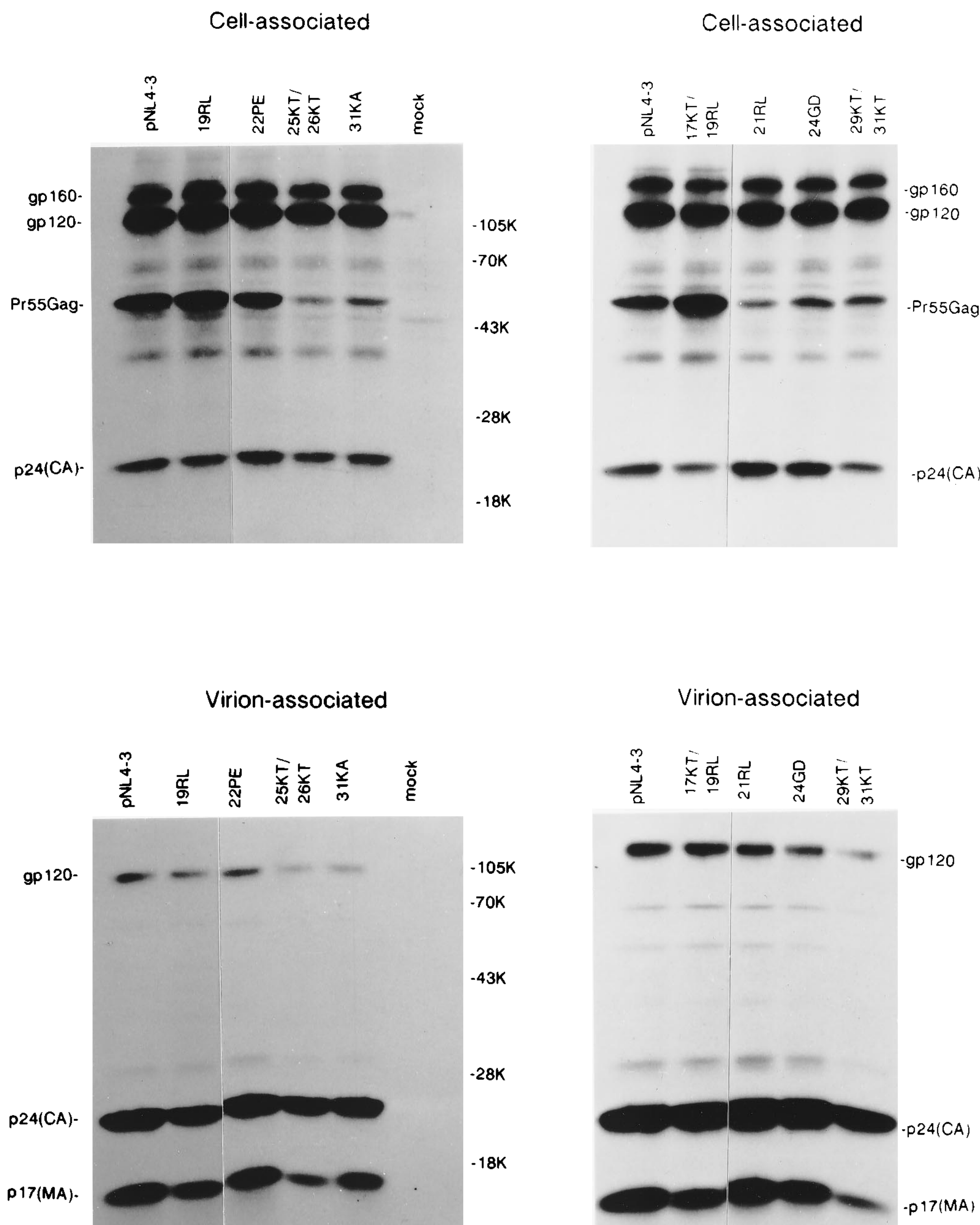

FIG. 2. Radioimmunoprecipitation analysis of mutant MA protein expression. HeLa cells were transfected with wild-type or MA mutant proviral clones and metabolically labeled with [^{35}S]Cys. Labeled cell supernatant was filtered and centrifuged to obtain virion pellets. Cell- and virion-associated material was lysed and immunoprecipitated with AIDS patient sera. The positions of the envelope glycoprotein precursor gp160, the mature surface envelope glycoprotein gp120, the Gag precursor Pr55^{Gag}, p24(CA), and p17(MA) are indicated. The mobilities of the molecular mass markers are shown in kilodaltons (K).

sented in Fig. 4. As expected, the differentiated human MDM cultures were refractory to infection by the T-cell line-restricted virus NL4-3 (13). Single- and double-amino-acid substitutions in the basic residues of the MA basic domain had only modest effects on infectivity in MDMs; this is consistent with our previous report that the 25KT/26KT mutant is able to initiate a spreading infection in primary human MDMs (13). We note that the basic residue mutations which caused the greatest reductions in macrophage infectivity (i.e., 25KT/26KT and 29KT/31KT) were those that impaired virus particle production in transfected HeLa cells (Table 1). The infectivity of the 29KT/31KT mutant in activated peripheral blood mononuclear cells from two different donors was similarly reduced compared with that of wild-type virus (data not shown).

Viruses containing mutations in both the basic domain of MA and in Vpr replicate in MDMs. It was previously suggested that MA and Vpr contain redundant nuclear localization domains (22). A prediction of this hypothesis is that MA basic domain mutations, which alone cause only modest reductions in virus replication in MDMs, would act in concert with a Vpr mutation to completely block replication in this cell type. To test this hypothesis, we generated HIV-1 preparations contain-

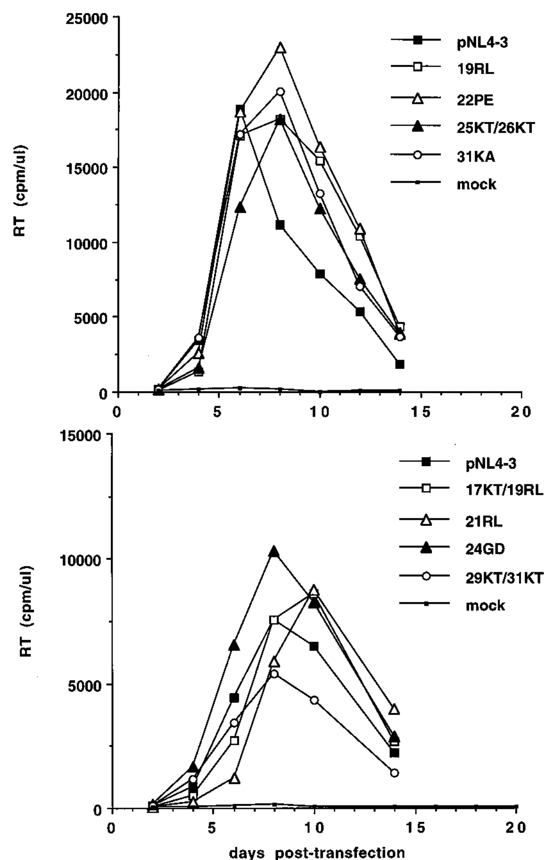

FIG. 3. Replication of MA basic domain mutants in the CEM(12D-7) T-cell line. The CEM(12D-7) T-cell line was transfected with wild-type or mutant proviral clones by the DEAE-dextran procedure (17). The cells were split 1:3 every 2 days posttransfection; aliquots were reserved for RT analysis at each time point.

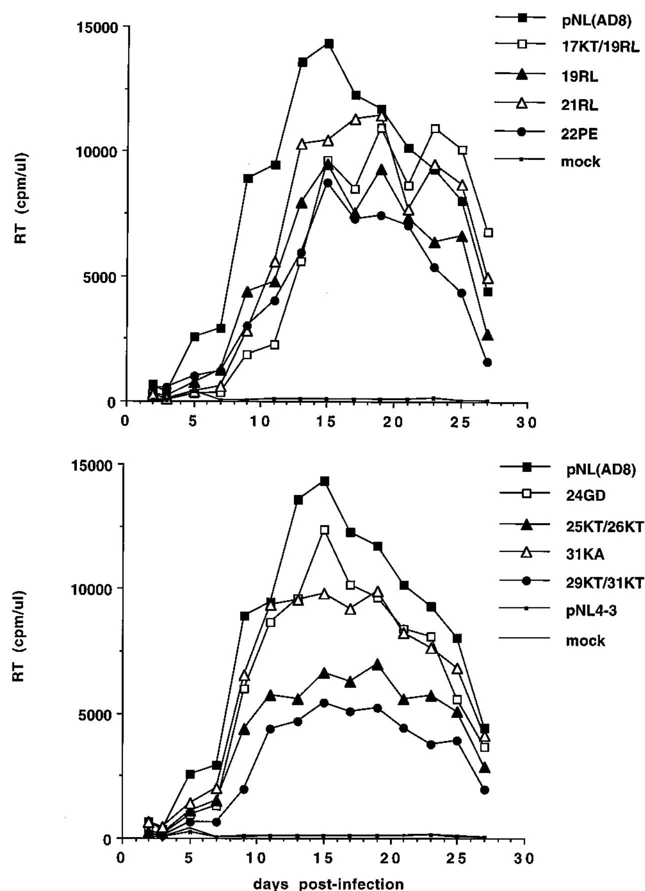

FIG. 4. Replication of MA basic domain mutants in MDM. Virus stocks were normalized for RT activity and were used to infect cultures of primary human MDMs. The medium was changed every 2 days; supernatants were reserved for RT analysis at each time point. The virus growth curves presented in this figure are derived from one infection but are plotted on two graphs to allow better visualization of the replication kinetics of each mutant.

ing mutations affecting both MA and Vpr by introducing a frameshift mutation at the *Eco*RI site in the *vpr* gene of pNL(AD8) and the 25KT/26KT and 31KA mutant derivatives of pNL(AD8). This was accomplished by digesting the pNL(AD8) and the MA mutant pNL(AD8) derivatives with *Eco*RI (*vpr* nucleotide 185 [32]), filling in the single-stranded ends with T4 DNA polymerase (New England BioLabs), and religating them with T4 DNA ligase (Boehringer Mannheim). The 25KT/26KT and 31KA MA mutations were selected for this analysis because they were previously reported to lose infectivity in primary human MDMs when present in combination with a *vpr* mutation (22, 43). Frameshifting of the *vpr* gene at the *Eco*RI site results in the truncation of the C-terminal 34 amino acids of Vpr. Previous studies have indicated that truncation of 17 amino acids from the C terminus of Vpr prevents its incorporation into virus particles (45), and truncation of 23 amino acids from the C terminus destabilizes Vpr (34).

Virus stocks of the Vpr mutant (R^-) and MA/Vpr double mutants (25KT/26KT/R^- and 31KA/R^-) were obtained by transfecting HeLa cells with the mutant proviral clones and were used to infect MDM cultures as described above. As shown in Fig. 5, the mutant containing a single mutation affecting the *vpr* gene (R^-) generated approximately two- to threefold less progeny virus than the wild type, pNL(AD8). Both of the MA/Vpr double mutants examined were still able to initiate spreading infections in primary MDMs, although the levels of particle production for the 31KA/R^- and 25KT/26KT/R^- mutants were approximately three- and sixfold reduced, respectively, relative to the wild type. Comparison of the effects of the single and double mutations on the levels of peak RT activity shown in Fig. 4 and 5 provides strong evidence that the combined MA and Vpr mutations are not synergistic but merely additive.

Conclusions. In this study, we analyze the effects of single- and double-amino-acid-substitution mutations in the highly basic domain of the HIV-1 MA. Our results indicate that (i) single- or double-basic-amino-acid-substitution mutations in the highly basic domain do not block infection of MDMs, (ii) a mutation in *vpr* causes a two- to threefold reduction in virus production during HIV-1 infection of MDMs but does not synergize with MA basic domain mutations in macrophage infection, and (iii) several basic domain mutations cause minor defects in virus particle production and envelope glycoprotein incorporation into virus particles.

Primary cells, including MDMs, from different donors frequently exhibit highly variable susceptibilities to HIV-1 infection (2, 7, 11). In MDMs from donors whose cells support only very limited HIV-1 replication, all mutations, including those disrupting the basic domain of MA, markedly reduce infectivity (data not shown), while in cells from other donors, which support efficient HIV-1 infection (e.g., Fig. 4), mutation of the

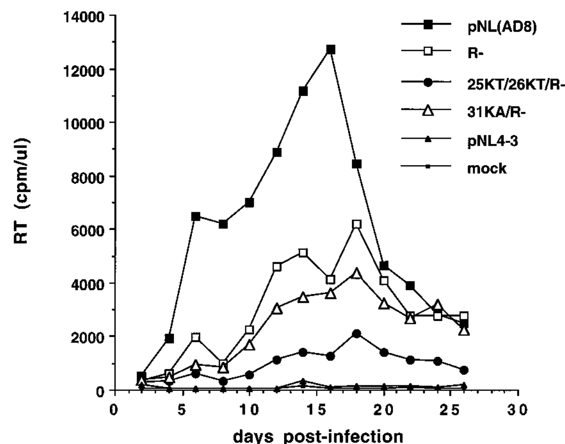

FIG. 5. Replication of MA/Vpr mutants in MDMs. Virus stocks were normalized for RT activity and used to infect cultures of primary human MDMs. The medium was changed every 2 days; supernatants were reserved for RT analysis at each time point.

basic residues only modestly impairs production of progeny virions. Thus, the defective phenotypes of HIV-1 mutants with alterations affecting a variety of different genes may be greatly exaggerated in relatively unsusceptible versus highly susceptible cells. For example, mutations in *vpu* or *vpr*, which generally have only modest effects on infectivity in MDMs (8, 24, 46), may profoundly impair virus replication when relatively unsusceptible cells from a particular donor are used (2, 11). This phenomenon is clearly illustrated in a recent report indicating that the reduction in virus production relative to the wild type resulting from a *vpr* mutation varied by more than 100-fold, depending on the donor from which the macrophages were derived (2). Our approach has been to evaluate the phenotype of HIV-1 mutants in peripheral blood mononuclear cells and MDMs obtained from multiple donors. We should note that mutations in proteins with presumably unrelated functions (i.e., in *vpu* and *vpr* or in *vpr* and *nef*) have also been reported to act synergistically to block HIV-1 replication in MDMs (2, 46). Thus, the appearance of synergy in the effects of mutations affecting two gene products is not sufficient to conclude that the two gene products share a related function.

The issue of infection efficiency may have some bearing on the apparent discrepancy between the results presented here and those reported by other investigators. For example, in the data presented by von Schwedler et al. (43), peak wild-type virus production in macrophage cultures was always approximately 10 ng of p24 per ml over a 100-fold range of multiplicities of infection. In the case of the highest multiplicity used, the virus yield approximated the input inoculum (20 ng of p24). More importantly, wild-type virion production in MDM cultures was 50-fold lower than peak wild-type virion production in the CEM T-cell line cultures (approximately 10 ng versus 500 ng of p24 per ml). In our experiments, comparable levels of virus production were evident in the MDM and CEM(12D-7) cultures (Fig. 3 versus Fig. 4). This difference in virus production is not likely to be due to differences in the state of macrophage differentiation in the two systems, because we maintained our cells in culture for 15 days before infection (as described in detail above), and a preculture period of similar length (10 to 15 days) was used by von Schwedler et al. (43). The relative virus production in MDM versus T-cell line cultures in the study by Heinzinger et al. is difficult to assess: the amount of virus produced in the T-cell line experiment was reported in nanograms of p24 per milliliter, whereas the yield from infected macrophages was reported as RT activity (22).

A large number of myristylated, membrane-bound proteins contain basic domains in positions analogous to that of the HIV-1 MA basic domain. Examples of such proteins include members of the Src family of tyrosine protein kinases (35) as well as other retroviral Gag proteins (i.e., murine leukemia virus, Mason-Pfizer monkey virus, feline leukemia virus, and others) (52). These basic domains are thought to act in concert with myristic acid to promote binding to cell membranes by associating with the acidic phospholipids that are concentrated on the cytoplasmic face of the lipid bilayer (35). Interestingly, several of the retroviruses with Gag proteins containing highly basic MA regions analogous to those of lentiviruses cannot infect nondividing cells. Thus, the presence of a basic domain near the N terminus of a retroviral Gag protein is not sufficient to permit infection of nondividing cells.

The results presented here do not exclude a role for the basic domain of MA in translocating the preintegration complex to the nucleus. It is possible, for example, that the elimination of the nuclear localization properties of MA requires the simultaneous removal of many or all of the basic residues in this region. Because the simultaneous mutation of a number of these basic residues blocks virus assembly (51, 52), the testing of this possibility becomes problematic. It is clear, however, that mutation of one or two basic residues in this domain, alone or in combination with a *vpr* mutation, does not block infection of MDMs.

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