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Dual Regulation of Silent and Productive Infection in Monocytes by Distinct Human Immunodeficiency Virus Type 1 Determinants

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The regulation of human immunodeficiency virus type 1 infection and replication in primary monocytes was investigated by mutagenesis of recombinant proviral clones containing an env determinant required for the infectivity of monocytes. Virus replication was assayed by determination of reverse transcriptase activity in culture fluids and by recovery of virus from monocytes following cocultivation with uninfected peripheral blood mononuclear cells. Three virus replication phenotypes were observed in monocytes: productive infection, silent infection, and no infection. Incorporation of the monocytotropic env determinant in a full-length clone incapable of infection or replication in primary monocytes (no infection) conferred the capacity for highly efficient virus replication in monocytes (productive infection). Clones with the env determinant but lacking either functional vpr or vpu genes generated lower replication levels in monocytes. Mutation of both vpr and vpu, however, resulted in nearly complete attenuation of virus replication in monocytes, despite subsequent virus recovery from infected monocytes by cocultivation with uninfected peripheral blood mononuclear cells (silent infection). These findings indicate a central role for the "accessory" genes vpu and vpr in productive human immunodeficiency virus type 1 replication in monocytes and indicate that vpu and vpr may be capable of functional complementation.

Human immunodeficiency virus type 1 (HIV-1) infection of macrophages has been demonstrated in brain, spinal cord, lung, lymph node, and skin during subclinical infection and disease and is postulated to underlie important clinical manifestations of HIV-1 infection, including disease latency and development of a spectrum of AIDS-related central nervous system disorders (2, 4, 11, 14, 15, 19, 33, 37). However, detailed molecular analysis of virus-host cell interactions involving monocytes was limited until recently by the restricted tropism of the earliest and most widely studied HIV-1 genetic clones for primary monocytes cultured in vitro (14, 15). Previously, we and others have demonstrated that a discrete env determinant, including the V3 loop but not the CD4-binding domain, is necessary and sufficient for HIV-1 infection of monocytes (23, 29, 36). Additionally, we have identified three virus replication phenotypes in monocytes in vitro, using molecularly defined proviral clones (35). These include productive infection, with the generation of high virus replication levels; silent infection, with low to undetectable virus replication in monocytes, despite ultimate virus recovery from infected monocytes following cocultivation with uninfected, phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs [lymphoblasts]); and no infection, with neither virus replication in nor virus recovery from monocytes observed. In the present study, we investigated the roles of the HIV-1 "accessory" genes vpr and vpu, which are dispensable for virus replication in primary and immortalized CD4+ T lymphocytes. We demonstrate that vpr and vpu are central to the regulation of virus replication in primary monocytes and together mediate the expression of silent versus productive infection.

To study viral regulation of monocyte infection, we utilized a panel of chimeric HIV-1 clones, constructed from the nonmonocytotropic clone HXB2 and the monocytotropic clone ADA, as previously described (16, 26, 35, 36). To correct a vpr defect in each of these clones, the result of a single base insertion in HXB2, 2.7-kb SalI-BamHI HXADA DNA fragments (nucleotides 5785 to 8474) were subcloned into the full-length proviral clone NL4-3, in which the vpr open reading frame is intact (1). The resultant NLHXA clones contained the ADA-derived env determinant previously localized to nucleotides 7040 to 7323, flanked by additional ADA- or HXB2-derived sequences encoding other portions of env and vpu and small portions of tat and rev. A clone in which the entire 5785-to-8474 sequence was HXB2 derived (thus lacking a monocytotropic env determinant) was used as a negative control for these experiments. Because HXB2 lacks a vpu initiator methionine codon, clones in which vpu was HXB2 derived were defective for that product, in contrast to clones with an ADA-encoded vpu. Finally, a vpr mutant corresponding to each NLHXADA clone was generated by introducing a frameshift mutation at codon 63.

Virions from the recombinant clones, generated by transformation, were assayed for their ability to infect and replicate in primary monocytes by the presence of reverse transcriptase (RT) activity in culture supernatants (25) and by the ultimate recovery of virus following cocultivation of monocytes with uninfected PBMCs. The results are summarized in Fig. 1. All clones containing the ADA-derived env determinants and an intact vpr gene generated high virus replication levels in monocytes. Inactivation of vpr in these
FIG. 1. Replication of recombinant HIV-1 clones with both wild-type and mutant vpr genes. (A) The panel of recombinant NLHXADA clones is represented diagrammatically. The region of the genome corresponding to the HXADA fragments (nucleotides 5785 to 8474) is expanded to highlight the relative positions of HXB2- and ADA-derived sequences. The open reading frames in this portion of the genome are represented above. Recombinant clones were generated by reciprocal DNA fragment exchanges of ADA- and HXB2-derived sequences into a SalI-BamHI fragment (5785 to 8474) from HXB2 subcloned into an intermediate shuttle vector, utilizing the restriction enzyme sites indicated on top. The resultant chimeric SalI-BamHI fragments were then subcloned into the clone NL4-3 to generate

<table>
<thead>
<tr>
<th>Clones</th>
<th>peak RT rescue vpr+</th>
<th>peak RT rescue vpr-</th>
</tr>
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<tbody>
<tr>
<td>NLHX</td>
<td>&lt;0.1</td>
<td>ND</td>
</tr>
<tr>
<td>NLHXADA-GG</td>
<td>7.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>NLHXADA-SM</td>
<td>14.0</td>
<td>5.4</td>
</tr>
<tr>
<td>NLHXADA-SK/GG</td>
<td>21.8</td>
<td>7.4</td>
</tr>
<tr>
<td>NLHXADA-KM</td>
<td>4.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>NLHXADA-KG</td>
<td>1.8</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>NLHXADA-G,M</td>
<td>2.1</td>
<td>&lt;0.1</td>
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clones, however, generated divergent results, depending upon the derivation of nucleotide sequences 5999 to 6345 (SK fragment). Clones in which this portion of the genome was ADA derived generated lower (but readily detectable) virus replication levels than did their wild-type vpr counterparts. However, vpr mutants in which SK was HXB2 derived typically failed to generate virus replication levels detectable above background in monocytes, despite subsequent virus recovery from these cultures onto uninfected PBMCs. The negative control clone, which carried a wild-type vpr but lacked the monocytotropic env determinant, generated virosomes which neither replicated in nor were recovered from monocytes, as previously demonstrated. No significant differences were seen in the replication of each virus strain on PBMCs obtained from different donors.

Monocytes were infected with recombinant HIV-1 clones containing a functional vpr gene, stained with toluidine blue, and examined by light microscopy (1-μm-thick plastic sections). Cultures infected with a nonmonocytotropic virus, NLHXADA-SK, which contains a functional vpu gene, were indistinguishable from uninfected cells, with rare, small multinucleated cells (Fig. 2A). Cultures productively infected with virus containing the monocytotropic env determinant, and a functional (NLHXADA-SM [Fig. 2B]) or nonfunctional (NLHXADA-GG [Fig. 2C]) vpu gene showed characteristic cytopathic effects (15). These consisted of the formation of multinucleated giant cells, often containing 10 or more nuclei per cell, and cell lysis. The frequencies and sizes of these cells were comparable in the NLHXADA-SM- and NLHXADA-GG-infected monocyte cultures. Virus production and cellular degeneration and necrosis were primarily confined to the multinucleated cells. Transmission electron microscopy examination demonstrated typical budding and mature virosomes in intracellular vacuoles that were associated with the plasma membrane, in both the presence and absence of vpu, but not in the NLHXADA-SK-infected cells (Fig. 2D). Freeze fracture scanning electron microscopy demonstrated budding of virion particles from the plasma membrane of monocytes infected with virus which lacked a functional vpu (Fig. 2E). No virus could be detected in monocytes infected with recombinant clones lacking both vpr and vpu (data not shown).

The SK fragment encodes the entire vpu gene product, 14 amino acids at the C termini of both the tat and the rev first exons, and the N-terminal 41 amino acids of env (Fig. 3). Although the absence of a vpu initiator methionine codon in HXB2 is the most obvious difference between the SK portions of HXB2 and ADA, a role for tat, rev, or env could not be ruled out. The env sequences differ at 7 of 41 predicted amino acid positions, not including the nonaligned insertion of 3 residues and deletion of 4 residues in ADA. All but three of these differences are confined to the signal peptide, which varies by up to 30% between different clones (20). Furthermore, tat and rev both differ at 3 of 14 amino acid positions between the ADA and HXB2 SK fragments, with four of these six changes being conservative in nature. Therefore, it is unlikely that these alterations in env, tat, or rev alter their function. However, to formally determine the specific requirement for vpu during HIV-1 infection of monocytes, the vpu initiator methionine codon of the silent infection clone NLHXADA-GG (vpr mutant) was restored by site-directed mutagenesis. The resultant clone was found to generate virus capable of productive infection of monocytes (data not shown).

HIV-1 and related lentiviruses are distinct from most other retroviruses in that besides the structural gag, pol, and env genes common to all retroviruses, they also encode a number of genes whose functions have been shown or are speculated to be regulatory in nature. In HIV-1, these genes include tat, rev, vif, nef, vpu, and vpr (6–8, 26, 32, 38). While tat, rev, and vif are essential for viral gene expression or virion infectivity, the precise role and overall importance of vpr, vpu, and nef are unclear, since these genes are dispensable for virus infection and replication in CD4+ lymphocytes in vitro (8–10, 12, 13, 22, 24, 30, 32). The availability of molecular HIV-1 clones which infect and replicate in monocytes at levels comparable to those observed with many monocytotropic virus isolates has facilitated investigation of the role that these viral genes may play in regulating the virus life cycle in monocytes. In the present study, we observed moderately decreased levels of virus replication in the absence of either vpr or vpu, whereas in the absence of both genes, virus replication in monocytes dropped to levels barely at or below the level of detection by the RT assay, such that infection of these cells usually could be detected only by virus rescue onto PBMCs.

The vpr open reading frame encodes a protein of 96 amino acids in most HIV-1 clones and is conserved in other lentiviruses, including visna-maedi virus (20, 31). Previous studies have shown that vpr is not required for HIV-1 infection or replication in CD4+ lymphocytic cell lines in vitro, although its inactivation led to slower replication kinetics and delayed cytopathogenicity in these cells (6, 10, 24). A recent study involving HIV type 2 (HIV-2) has shown that vpr is likewise dispensable during infection of PBMCs and T-cell lines but essential for productive infection of monocytes (17). The vpr protein has been demonstrated by radioimmunoprecipitation to be virion associated, and thus it is speculated to function either late in the virus life cycle, during particle assembly or maturation, or early, during the initial stages of infection (6). The vpu gene encodes an 80- to 82-amino-acid protein. It has not been reported whether the vpu protein is found in virion particles. vpu has been shown to augment virion particle release from infected cells without affecting levels of viral RNA or protein synthesis (8, 32). In the absence of vpu, a higher ratio of immature to mature particles has been seen, with a shift in capsid...
form from the plasma membrane to intracellular membranes (8). In monocytes, however, particle assembly and release occur both at the plasma membrane and in intracellular vacuoles in the presence or absence of vpu, as shown in Fig. 2D.

It is intriguing that HIV-2 and simian immunodeficiency virus lack a vpu open reading frame but instead carry a gene designated vpx, which encodes a protein of 114 to 118 amino acids in these viruses (20). vpu and vpx occupy similar positions in their respective viral genomes, between pol and env, but have only distant amino acid homology. Recently, it has been suggested that vpx and vpr arose by duplication from a common progenitor in HIV-2 and simian immunodeficiency virus, on the basis of predicted amino acid sequence homology between the genes (34). To investigate the possibility of a similar link between vpr and vpu in HIV-1, the predicted amino acid sequences of both vpu and vpx were aligned with that of vpr (Fig. 4). Although less compelling that the homology between vpr and vpx, a 38% identity was observed between vpr and vpu over a 24-residue overlap at the C terminus of vpu and the N terminus of vpr. These sequences were particularly rich in acidic residues. Similarity in the hydrophilicity profiles of these portions of the vpu, vpr, and vpx products was also noted. The striking effect on virus replication levels in monocytes observed only when both genes were defective suggests that their gene products may perform similar roles and thus provide partial functional complementation. Alternatively, since lower replication levels were observed in the absence of either gene, the nearly complete attenuation observed in the absence of both may result from a compound effect of the loss of two relatively important but functionally unrelated genes. More detailed studies to determine the precise mechanisms of action of the vpr and vpu gene products will be required to address these alternatives. In either case, our data indicate that together, vpr and a second determinate, vpu, are more important for efficient HIV-1 infection and replication in primary monocytes than was observed previously in lymphocytes. These observations provide a rationale for designing potential antiviral therapies to block the action of these gene products during HIV-1 infection of monocytes.

Persistent infection of tissue macrophages plays an important role in the pathogenic effects of other lentiviruses, including equine infectious anemia virus, visna-maedi virus, and caprine arthritis-encephalitis virus, providing a sanctuary for continuous virus replication in the face of a vigorous host immune response (15, 21). The onset of increased virus replication has been correlated with the onset of clinical disease manifestations, such as encephalitis, pneumonitis, arthritis, and hemolytic anemia. Similarly, HIV-1 infection of macrophages generates a reservoir of virus which is present throughout the course of subclinical infection and clinical disease. The existence of poorly replicative HIV-1 variants may be essential for establishment of persistent macrophage infection during the early, asymptomatic stage of disease. Several studies have suggested a relationship between the level of HIV infection in macrophages and the degree of clinical disease.

FIG. 3. Comparison of SK virus replication determinant from HXB2 and ADA. The predicted amino acid sequences of vpu and the portions of tat, rev, and env which are encoded by nucleotides 5999 to 6345 (SK fragment) from HXB2 and ADA are aligned by using single-letter amino acid designations. Identical residues are indicated within boxes.
between the in vitro replicative properties of HIV-1 isolates in T lymphocytes and clinical-disease stage, with earlier isolates tending to replicate more slowly and to lower levels ("slow, low") than isolates from later stages of disease ("rapid, high") (3, 5, 27). Nonessential regulatory genes are ideally suited to act as "molecular switches" for control of replication phenotypes by their activation or inactivation, particularly in viruses such as HIV-1, which characteristically generate high levels of sequence diversity. We demonstrate here that discrete genetic alterations in such accessory genes result in profoundly different replication rates in monocytes in vitro, which suggests a mechanism for transition from subclinical to clinical disease in vivo. These findings thus provide a rationale for addressing on a wider scale whether functional status of vpr and/or vpu correlates with disease stage or serves as a potential prognostic indicator of disease progression and outcome.

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