Gp120 stability on HIV-1 virions and Gag-Env pseudovirions is enhanced by an uncleaved Gag core

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Abstract

Human immunodeficiency virus type-1 (HIV-1) particles incorporate a trimeric envelope complex (Env) made of gp120 (SU) and gp41 (TM) heterodimers. It has been previously established that soluble CD4 (sCD4) interaction leads to shedding of gp120 from viral particles, and that gp120 may also be easily lost from virions during incubation or particle purification procedures. In the design of HIV particle or pseudovirion-based HIV vaccines, it may be important to develop strategies to maximize the gp120 content of particles. We analyzed the gp120 retention of HIV-1 laboratory-adapted isolates and primary isolates following incubation with sCD4 and variations in temperature. NL4-3 shed gp120 readily in a temperature- and sCD4-dependent manner. Surprisingly, inactivation of the viral protease led to markedly reduced shedding of gp120. Gp120 shedding was shown to vary markedly between HIV-1 strains, and was not strictly determined by whether the isolate was adapted to growth on immortalized T cell lines or was a primary isolate. Pseudovirions produced by expression of codon-optimized gag and env genes also demonstrated enhanced gp120 retention when an immature core structure was maintained. Pseudovirions of optimal stability were produced through a combination of an immature Gag protein core and a primary isolate Env. These results support the feasibility of utilizing pseudovirion particles as immunogens for the induction of humoral responses directed against native envelope structures.

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Introduction

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein complex (Env) consists of two subunits, the surface subunit gp120 (SU) and the transmembrane subunit gp41 (TM). Env is synthesized as a single glycoprotein precursor, gp160, and is assembled into oligomers (Pinter et al., 1989; Schawaller et al., 1989). These envelope oligomers have been shown to be trimeric complexes on the surface of cells and on viral particles (Chan et al., 1997; Kwong et al., 2000). During exocytic transport to the plasma membrane, the gp160 precursors are cleaved at a conserved site by cellular furin-like proteases (Earl et al., 1990; Willey et al., 1988). The SU-TM heterodimers that make up the heterotrimeric complex are linked by noncovalent interactions (Willey et al., 1988). The envelope protein complex plays a key role in mediating viral tropism through the specific binding of gp120 to the receptor CD4 molecule and to coreceptor molecules on the cell surface (Clapham et al., 1991; Dalgleish et al., 1984; Klatzmann et al., 1984; Lasky et al., 1987; Maddon et al., 1986). Following receptor and coreceptor binding, the fusion peptide region of TM interacts with the target membrane, and conformational changes occur in the TM subunit that lead to apposition of the viral and cellular membranes (Bosch et al.,
Recombinant, soluble CD4 (sCD4) is capable of binding to HIV-1 gp120 in a manner similar to that of full-length CD4. sCD4 consists of the two N-terminal immunoglobulin-like domains of the ectodomain of CD4, and can neutralize HIV-1 infectivity and inhibit virus-induced syncytium formation (Deen et al., 1988; Fisher et al., 1988; Hussey et al., 1988; Trauenecker et al., 1988). sCD4-dependent HIV-1 neutralization can occur through two mechanisms. First, sCD4 can interact with the CD4 binding site on gp120 and thereby prevent gp120 from binding to receptor CD4 molecules on the target cell membrane (Laskey et al., 1987). Second, sCD4 has been demonstrated to induce the nonreversible loss of gp120 from viral particles (Hart et al., 1991; McKeating et al., 1991; Moore et al., 1990, 1991b). Gp120 loss for lab-adapted strains of HIV-1 occurs in a sCD4 concentration-dependent manner (Hart et al., 1991; Kirsh et al., 1990; Moore et al., 1990). It has also been shown that the HIV-1 envelope glycoprotein complex is dissociated from HIV particles in response to elevations of temperature or extremes of pH (Fu et al., 1993), even in the absence of sCD4 interaction.

Primary isolates of HIV-1 differ markedly from isolates that have been adapted to growth on immortalized T cell lines. Primary HIV-1 isolates are relatively resistant to neutralization by polyclonal sera from HIV-1 infected individuals and resist neutralization by many monoclonal antibodies that neutralize laboratory-adapted HIV-1 strains (Mascola et al., 1994; Matthews, 1994; Moore et al., 1995). sCD4 also readily neutralizes laboratory-adapted strains, while primary isolates require much higher concentrations of sCD4 to obtain similar levels of neutralization (Ashkenazi et al., 1991; Daar et al., 1990; Moore et al., 1993, 1992; O’Brien et al., 1994). These data suggest that neutralization epitopes exposed on gp120 molecules on the surface of laboratory-adapted isolates are normally concealed in primary isolate viruses, and imply that one adaptation to T cell line replication is conformational alteration of gp120 to a more neutralization-friendly state. The enhanced epitope exposure on laboratory-adapted isolates may be due to adaptive changes in Env that optimize the CD4–Env interaction in the absence of selective pressure produced by the humoral immune response.

Attempts to elicit humoral responses against HIV using recombinant envelope glycoprotein molecules have thus far failed to elicit strong cross-reactive neutralizing antibodies against primary isolates (Cohen, 1993; Mascola et al., 1996; Sawyer et al., 1994). Responses against recombinant gp120 molecules are generally strongest against regions of gp120 that are occluded in the native trimer, and are thus not capable of neutralization of the viral particle (York et al., 2001). Strategies to overcome this include the engineering of molecules that more closely resemble the native trimeric complex through introduction of disulfide bonds for gp120-gp41 stabilization (Binley et al., 2000; Sanders et al., 2000), disruption of the SU/TM cleavage site through mutagenesis (Earl et al., 1994; Stamatatos et al., 2000; VanCott et al., 1997), or through the engineering of heterologous leucine zipper domains into gp41 to promote trimer formation (Yang et al., 2000, 2002). An oligomeric, V2-deleted version of the SF162 glycoprotein was recently shown to elicit antibodies in macaques with neutralizing activity against a limited number of HIV-1 primary isolates (Barnett et al., 2001). Another approach to this problem is to design HIV pseudovirions composed of Gag and Env proteins (Gheysen et al., 1989; Haffar et al., 1990). Pseudovirions allow the native envelope trimer to be presented on a noninfectious particle platform. Promising results with pseudovirions have been reported in the literature, including the ability of Env on pseudovirions to elicit neutralizing antibodies against a homologous primary isolate virus (Montefiori et al., 2001). The production of pseudovirions that incorporate Env complexes in an efficient and stable manner is thus a worthwhile goal for vaccine design.

In this study, we examined the retention of gp120 on laboratory-adapted HIV-1 isolates and primary isolates following sCD4 addition and temperature variation. We then examined HIV-1 pseudovirions incorporating primary isolate envelope glycoproteins for their gp120 retention under similar conditions. We demonstrate that NL4-3 virions with an uncleaved core have an increased retention of gp120 following sCD4 addition and exposure to increasing temperatures. Differences in gp120 shedding among different isolates were strain specific, and did not strictly correlate with lab-adapted vs primary isolate classification. Finally, we show that HIV-1 pseudovirions incorporating primary isolate envelope glycoproteins do not readily shed gp120, and that gp120 retention on pseudovirions can also be optimized through the use of an immature Gag core. These results support the design of pseudovirion immunogens capable of presenting native Env complexes to elicit humoral immune responses.

**Results**

*Effect of sCD4 and temperature increases on gp120 shedding by NL4-3 virions*

Previous data have demonstrated that sCD4 leads to shedding of gp120 from lab-adapted strains of HIV-1. In order to determine the effect of sCD4 interaction on envelope stability, concentrated NL4-3 virions were incubated with increasing concentrations of sCD4 (0-10 μg/mL) for 2 h at 0, 4, and 37 °C. These samples were then purified by centrifugation through a sucrose cushion, and the particle-associated envelope concentration was determined using a gp120 capture ELISA. A significant dissociation of gp120 from NL4-3 virions was observed with increasing temperature and with increases in sCD4 concentration (Fig. 1A). The dissociation of gp120 from NL4-3 virions in the pres-
Fig. 1. Effect of sCD4 concentration and temperature elevation on NL4-3 virion envelope shedding. Virions were incubated with increasing sCD4 concentrations at 0 (closed squares), 4 (open circles), and 37 °C (closed triangles) for 2 h. Virus particles were subsequently purified by centrifugation through a sucrose cushion, and the virion gp120 content determined using a gp120 capture ELISA. Envelope shedding was calculated as percent virion bound gp120 remaining in the postincubation viral pellet. 100% retention was set as the amount of gp120 retained at 0 °C in the absence of sCD4. Results are representative of three independent experiments. (A) NL4-3 virion gp120 retention with increasing sCD4 concentration. (B) NL4-3 gp120 retention seen with increasing temperature in the absence of sCD4. Results are representative of three independent experiments. (C) NLPr  virion gp120 retention with increasing sCD4 concentration. (D) NLPr  gp120 retention at differing temperatures in absence of sCD4. (E) NL4-3 virions harvested from H9 cell supernatants were examined for gp120 retention in an identical manner to that in A. (F) NL4-3 virions harvested from H9 cell supernatants examined for gp120 retention at increasing temperatures in the absence of sCD4.
ence of sCD4 was biphasic, with the steepest dissociation occurring between 0 and 1 μg/ml sCD4. NL4-3 virions shed significant amounts of gp120 as a result of sCD4 binding at all temperatures tested. Maximal shedding of gp120 was observed at 37 °C with 10 μg/mL sCD4, which resulted in greater than 80% dissociation from NL4-3 virions (Fig. 1A, grey diamonds). It is interesting to note that gp120 was even shed from NL4-3 virions at 0 °C in a sCD4 dependent manner (Fig. 1A, filled squares). Gp120 was also lost from NL4-3 virions in a temperature-dependent manner in the absence of sCD4, indicating that heat alone can lead to significant gp120 loss from this isolate (Fig. 1B). A significant loss of gp120 was seen for virions incubated at 4 °C, as compared with virions that were strictly maintained in liquid ice (0 °C, Fig. 1B). When increasing temperatures were employed, an additional increment in gp120 shedding was not demonstrated until the temperature was increased to 50 °C or higher.

The degree of shedding of gp120 from NL4-3 virions in response to temperature increase and interaction with sCD4 may be detrimental to particle-based vaccine strategies. To test the hypothesis that an immature Gag core may act to stabilize gp120 on the virion, as suggested by a recent report (Wyma et al., 2000), we performed identical experiments with an NL4-3 provirus containing inactivating substitutions of the protease active site (NLPr−). Maximal shedding of gp120 from NLPr− virions was observed at 37 °C and 10 μg/mL sCD4, but resulted in only a 23% loss of gp120 (Fig. 1C). Loss of gp120 seen with NLPr− virions was remarkably less at all temperatures and sCD4 concentrations than that seen with NL4-3 (compare Fig. 1A and C). NLPr− virions also displayed enhanced stability to temperature increases in the absence of sCD4, with no substantial loss until incubated at temperature of greater than or equal to 50 °C (Fig. 1D). We conclude that the immature Gag core resulted in a significant enhancement of the stability of gp120 on virions.

We noted during these experiments that the total amount of gp120 on virions produced by transfection of 293T cells exceeded that of virus grown in the cell line H9 (Table 1). To determine if the mode of production or amount of incorporated gp120 accounts for the substantial shedding observed for NL4-3 virions, we analyzed the stability of gp120 on virus that was grown in H9 cells. NL4-3 grown in H9 cells rapidly lost gp120 in a temperature- and sCD4-dependent manner, with a 60% loss of gp120 at 37 °C and 10 μg/ml sCD4 (Fig. 1E). Gp120 was also shed in a temperature-dependent manner in the absence of sCD4 (Fig. 1F). Results with H9-derived virus were thus similar to those seen with virus produced by transfection, although slightly less in degree. We conclude that a small portion of the extreme amount of gp120 loss shown in Fig. 1A could be attributable to the virus production method, but that NL4-3 produced by either method demonstrates significant shedding. Taken together, these results indicate that NL4-3 readily sheds gp120 unless stabilized through the presence of an uncleaved Gag core.

The gp120 ELISA used in our experiments was carefully validated using a series of laboratory-adapted and primary isolate viruses and recombinant proteins (data not shown). However, in order to further validate our results concerning the enhancement of envelope complex stability by the immature virion core, we analyzed NL4-3 and NLPr− virions using autoradiographic analysis of particles incubated under varying conditions of temperature and concentrations of sCD4. Visual inspection of gp120 bands present on the autoradiograph from this experiment indicates that shedding occurred in a sCD4-and temperature-dependent manner (Fig. 2A). Less gp120 shedding was apparent for NLPr− (Fig. 2B). To quantify the differences observed, phosphorimager analysis was performed on the gels from this experiment. These experiments revealed that NL4-3 virions lost 75% of gp120 when incubated with 10 μg/mL sCD4 at 37 °C (Fig. 2C). NLPr− virions demonstrated enhanced stability of particle-incorporated gp120 as compared with NL4-3 virions at both 0 and 37 °C (Fig. 2D). It is important to note that the relative amounts of gp160 on viral particles of NL4-3 or NLPr− particles were roughly similar, indicating that retention of uncleaved Env was not responsible for the differences seen in shedding of gp120 by the ELISA analysis. This analysis is in agreement with the ELISA data presented in Fig. 1, and further supports the finding that uncleaved core stabilizes gp120 on virions.

### Stability of gp120 on primary HIV-1 isolates vs laboratory-adapted isolates

The profound shedding of gp120 observed for NL4-3 supported previous work indicating that this property is common to laboratory-adapted HIV isolates (Groenink et al., 1995; O’Bien et al., 1994). We next sought to confirm the finding that primary isolates of HIV retain gp120 in an enhanced manner, as this would suggest that primary isolate Env may be desirable on pseudovirion-based immunogens.

### Table 1: Gag:Env ratio of HIV-1 virions and pseudovirions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gag:Env Ratio</th>
<th>Tropism</th>
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<tbody>
<tr>
<td>NL4-3</td>
<td>71:1</td>
<td>X4</td>
</tr>
<tr>
<td>NL4-3(H9)</td>
<td>323:1</td>
<td>X4</td>
</tr>
<tr>
<td>NLPr−</td>
<td>2:1</td>
<td>X4</td>
</tr>
<tr>
<td>S162</td>
<td>45:1</td>
<td>R5</td>
</tr>
<tr>
<td>Bal</td>
<td>908:1</td>
<td>R5</td>
</tr>
<tr>
<td>MN</td>
<td>66:1</td>
<td>X4</td>
</tr>
<tr>
<td>Pseudovirions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gag-Pro/BaL</td>
<td>384:1</td>
<td>R5</td>
</tr>
<tr>
<td>55Gag/BaL*</td>
<td>70:1</td>
<td>R5</td>
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* Inefficient capture of Pr55Gag by p24 capture ELISA resulted in artificial lowering of Gag:Env ratio.
We chose to examine the extent of gp120 shedding exhibited by two primary isolates, SF162 and BaL. These viruses were grown in PBMCs, concentrated by centrifugation through a sucrose cushion, and examined using the envelope stability assay already described. Indeed, gp120 on these isolates was remarkably stable following sCD4 addition and temperature increases (Fig. 3A–D). BaL gp120 was stable to sCD4 addition up to 10 μg/mL at 37 °C (Fig. 3A). BaL also retained nearly 100% of its gp120 upon incubation in the absence of sCD4; shedding was only evident at temperatures at or exceeding 50 °C (Fig. 3B). Similar results were obtained from the examination of the primary HIV-1 isolate SF162 upon incubation with sCD4 (Fig. 3C) or upon incubation at a range of temperatures (Fig. 3D). The reason for the measured increase in pelleted gp120 at 10 μg/ml sCD4 over baseline values for this isolate is not yet clear. This apparent increase was present on repeated experiments, and could be due to some enhancement of viral pelleting upon addition of high concentrations of sCD4. Overall, these results are consistent with previous reports indicating that primary HIV-1 isolate envelope glycoprotein complexes are relatively resistant to sCD4-induced shedding as compared to lab-adapted strains (Moore et al., 1992, 1991; O’Brien et al., 1994).

The stability of gp120 on primary isolates, together with the instability of gp120 on NL4-3 virions, suggested that primary isolates retain gp120 in a much more stable manner than lab-adapted strains. However, a recent study reported that gp120 on several lab-adapted strains was also quite stable (Chertova et al., 2002). To determine if the shedding of gp120 observed with NL4-3 virions could be generalized to other lab-adapted isolates, we examined the effect of

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Fig. 2. Autoradiographic analysis of sCD4-induced shedding of gp120 from NL4-3 virions. Virions were labeled with [35S]cysteine/methionine and incubated with the sCD4 concentrations indicated for 2 h at 0 and 37 °C. Virion-associated proteins were immunoprecipitated using HIV patients' sera and analyzed by SDS-PAGE and autoradiography. (A) NL4-3 virions. (B) NLPr virions. (C) Phosphorimager quantitation of gp120 from NL4-3 virion results presented in A. The levels of gp120 present in each lane are presented as a percentage of virion-bound gp120 in the absence of sCD4 treatment. (D) Phosphorimager quantitation for NLPr virions.
Fig. 3. Effect of sCD4 concentration and temperature elevation on HIV-1 primary isolates and lab-adapted isolates. Gp120 retention following exposure to increasing concentrations of sCD4 (left panels) or increasing temperatures in the absence of sCD4 (right panels) was examined by methods identical to those in Fig. 1. (A) Gp120 retention for primary isolate SF162 with sCD4 exposure. (B) SF162 gp120 retention at varying temperatures in absence of sCD4. (C) HIV-1 primary isolate BaL gp120 retention with sCD4 exposure. (D) BaL gp120 retention at increasing temperatures in absence of sCD4. (E) Lab-adapted HIV-1 isolate MN gp120 retention with increasing sCD4. (F) MN gp120 retention at increasing temperatures.
temperature variation and sCD4 interaction upon the gp120 content of the lab-adapted strain MN. MN was found to be resistant to sCD4-induced shedding of gp120 up to sCD4 concentrations of 10 μg/ml at 37 °C (Fig. 3E). Furthermore, MN retained its envelope glycoproteins when subjected to temperature elevations up to 37 °C. MN, like the primary isolates examined, begin to shed gp120 only at or exceeding 50 °C. These results indicate that both lab-adapted and primary isolate envelope glycoprotein complexes can exhibit stability to sCD4 and kinetic energy increase. Because NL4-3 shed gp120 readily, while MN did not, it cannot be concluded that all lab-adapted strains of HIV-1 are prone to sCD4-induced shedding of gp120.

Gag-Env ratio on virions and pseudovirions

We considered the possibility that the total amount of gp120 on virions and pseudovirions may relate to gp120 retention. For example, if NL4-3 produced by transfection of 293T cells incorporated much higher amounts of gp120 than primary isolates, it could have some aberrantly incorporated or “loosely-associated” gp120 that is more readily shed. To test this hypothesis, we measured the gp120 and p24 content of each of the purified virion and pseudovirion particles employed in our studies (Table 1). NL4-3 produced in 293T cells was found to have a molar ratio of Gag-to-Env of 71:1. This is very similar to the 60:1 Gag-to-Env ratio determined by Chertova and co-workers (Chertova et al., 2002), and also agrees with an earlier report for T cell line adapted viruses in which ratios between 70:1 and 297:1 were observed (O’Brien et al., 1994). Therefore, the amount of gp120 on the surface of the virus derived from 293T cells was not excessive, and this should not account for gp120 shedding. NL4-3 virions purified from H9 supernatants had a four- to fivefold decrease in gp120 as compared with those from 293T (Table 1), yet the shedding of gp120 was comparable (Fig. 1C). We found that Gag-to-Env ratios varied from 45:1 (SF162) to 908:1 (BaL) for the viruses used in our study. The ratios determined for NLPr− and Pr55Gag/BaL are unfortunately not comparable, as we found that the uncleaved Gag was inefficiently captured by our p24 ELISA. However, it is clear that the amount of Env on the particle surface was unrelated to the categorization of a virion as lab-adapted or primary isolate in origin. It is also evident that stability of gp120 on the particle surface does not correlate with Gag-to-Env ratio.

Gradient analysis of virion and pseudovirion gp120 incorporation

We performed sucrose density gradient analysis in order to demonstrate that the gp120 measured in shedding experiments was present on virion or pseudovirion particles. Metabolically labeled virions and pseudovirions were separated by density centrifugation and then immunoprecipitated using HIV+ patient sera prior to analysis by SDS-PAGE and autoradiography. Note that the exposure times were designed to demonstrate the gp120 bands clearly, resulting in oversaturation of p24 or Pr55Gag signal on these autoradiograms. All particles peaked at the accepted retroviral density of 1.16–1.18 g/mL (Fig. 4A–D). A majority of the particle-associated envelope glycoprotein seen on both virions and pseudovirions was present in the cleaved (gp120) form. The amount of gp120 incorporated on pseudovirions as assessed by Env/Gag ratios was quite similar to that of HIV-1 virions (Fig. 4 and Table 1). Little gp120 was found in gradient fractions other than those of retroviral particle density, indicating that the gp120 measured in this study was not significantly confounded by measurement of aggregates of gp120 or of gp120 on microvesicles.

Effect of sCD4 addition and heat on stability on Gag-Pro-Env and Gag-Env pseudovirions

Pseudovirion-based vaccines should be able to present HIV-1 envelope glycoproteins in a native, oligomeric state, and thus represent a promising vaccine strategy. We produced both mature and immature HIV-1 pseudovirions incorporating BaL Env through transfection of codon-optimized Gag and Env expression plasmids. Mature (cleaved) pseudovirions were produced through expression of Gag and protease, and are referred to as Gag-Pro/BaL pseudovirions, while immature ( uncleaved) pseudovirions were produced by expression of Pr55Gag in the absence of protease, and are referred to as Pr55Gag/BaL pseudovirions. Pseudovirions were then examined for gp120 stability in experiments similar to those already described. Gag-Pro/BaL pseudovirions were relatively resistant to sCD4 addition at the temperatures tested, shedding a maximum of 22% at 10 μg/mL sCD4, 37 °C (Fig. 5A). In response to increasing temperatures alone, the Gag-Pro/BaL particle shed only 20% of its envelope glycoprotein, and shed 50% of gp120 when the temperature was raised to 50 °C (Fig. 5B). Pr55Gag/BaL pseudovirions demonstrated enhanced gp120 stability as compared to mature particles containing the same envelope. This finding was most apparent in the retention of gp120 at increasing temperatures (compare Fig. 5B and D), but was also seen to a small degree with increasing sCD4 concentrations (compare 4 °C curves in Fig. 5A and C). These results support the differential shedding of gp120 observed with NL4-3 and NLPr−. The smaller difference observed in retention of gp120 seen in this case is likely due to the inherently more stable primary isolate BaL Env employed. Importantly, these results suggest that pseudovirion vaccine designs may benefit in gp120 stability from the use of an immature Gag core combined with a primary isolate HIV-1 envelope glycoprotein.
Discussion

HIV-1 virus-like particles or pseudovirions represent an attractive vehicle for the presentation of native, trimeric Env complexes to the immune system. The present study was initiated in order to understand the factors that lead to gp120 retention or loss from virions and pseudovirions, in order to design enhanced pseudovirion-based vaccines. It has been well established that gp120 is shed from virions following a variety of manipulations, including exposure to CD4, increases in temperature, and purification procedures (Fu et al., 1993; Groenink et al., 1995; McKeating et al., 1991a,b; Moore et al., 1992; O’Brien et al., 1994). An early attempt to produce an HIV vaccine through the inactivation and purification of HIV particles was severely limited by loss of gp120 during the particle purification process, including exposure to CD4, increases in temperature, and purification procedures (Fu et al., 1993; Groenink et al., 1995; McKeating et al., 1991a,b; Moore et al., 1992; O’Brien et al., 1994). An early attempt to produce an HIV vaccine through the inactivation and purification of HIV particles was severely limited by loss of gp120 during the particle purification process, although the gp120-depleted product has been further pursued as an immunogen for T cell responses to Gag (Trauger et al., 1994). If one objective of a pseudovirion-based vaccine is to produce neutralizing antibody responses against HIV, then limiting the loss of gp120 from the pseudovirion surface is a critical goal.

Our results using NL4-3 are consistent with previous reports indicating that lab strains of HIV are prone to shed gp120. We demonstrated a dramatic loss of gp120 upon exposure to sCD4 for this isolate. Kinetic energy was essential in determining the magnitude of gp120 dissociation during sCD4 incubation. We note for the first time a substantial difference between 0 °C (samples on wet ice) and a slight increase in kinetic energy to 4 °C (Fig. 1), although even greater loss was seen at 37 °C. A substantial loss of gp120 was also noted following incubation at increasing temperatures in the absence of sCD4, suggesting that there is an inherent instability of gp120 on NL4-3 virions. In contrast to NL4-3, primary isolates demonstrated very little shedding of gp120 following sCD4 exposure, and retained substantial amounts of gp120 when incubated at temperatures up to 50 °C. These results were also seen with pseudovirions produced by expression of codon-optimized gag and env genes.

We expected that instability of gp120 might be a general property of laboratory-adapted viral isolates as suggested by previous work (Moore et al., 1992, 1990). However, we found that the laboratory-adapted isolate MN retained gp120 quite efficiently. We interpret this finding to mean that gp120 loss or retention properties vary by isolate, and not necessarily by adaptation to growth on T cell lines. Support for this interpretation comes from a recent report (Chertova et al., 2002). These investigators used HPLC to quantitate gp120 and gp41 on purified virions, and found that little or no SU was shed from both primary isolate and lab-adapted viruses upon exposure to heat or sCD4. As in our study, MN did not demonstrate any significant gp120
shedding. Thermal stability of gp120 on MN was also demonstrated and agrees with the findings reported here. In contrast, NL4-3 was examined only by immunoblot in the Chertova et al. report, and the blot was interpreted as indicating a lack of gp120 shedding. Our data clearly show that NL4-3 produced by transfection of 293T cells or grown in H9 cells readily sheds gp120. We conclude from these data that there are strain-specific differences in gp120 stability on virions, and that for some isolates (such as NL4-3) gp120 shedding can be profound.

It is important to note that the gp120 retention assay utilized in this study measured virion-associated gp120 as the amount of gp120 present on virions following pelleting through 20% sucrose (before and after temperature incubations or sCD4 exposure). We chose this method to exclude from the analysis any gp120 that was already dissociated from virions at the time of supernatant collection. It is possible that some virion-associated gp120 was lost in the initial pelleting step, and that this could result in an underestimation of gp120 shedding. However, the amount of gp120 we detected on the pelleted virions was consistent with the findings of other investigators for purified HIV particles (Chertova et al., 2002), suggesting that our treatment was not overly harsh. In addition, our results with NL4-3 demonstrate that large degrees of shedding were measured in a very reproducible manner. We conclude that the pelleting assay is a valid means of measuring gp120 stability on HIV virions and pseudovirions.

Fig. 5. Effect of sCD4 on pseudovirion-associated gp120 retention. (A) Pseudovirions produced by expression of Gag, protease, and BaL gp160 expression were examined for gp120 retention after incubation with increasing concentrations of sCD4 using methods identical to those for Fig.1. (B) Retention of gp120 following incubation at increasing temperature for Gag-pro/BaL gp160 pseudovirions. (C) Pseudovirions with an immature core (Pr55Gag and BaL gp160 expression) were analyzed for gp120 retention with increasing concentrations of sCD4. (D) gp120 retention with increasing temperature for Gag/BaL gp160 pseudovirions.
The most striking finding from this report is that an uncleaved Gag core contributed substantially to gp120 stability on HIV-1 particles. We hypothesize that the enhanced envelope stability of NLPr− virions is due to interactions between Gag and the cytoplasmic tail of gp41. An uncleaved gag core may enhance gp120 retention by providing conformational constraints on the ectodomain of the HIV-1 envelope glycoprotein, leading to enhanced gp120 stability. This hypothesis is supported by a recent report showing that immature viral cores, but not mature cores, retain gp120 following purification by TX-100 extraction and centrifugation on sucrose gradients (Wyma et al., 2000). The association of gp120 with the viral core in this study required the gp41 cytoplasmic tail, and the stable retention of gp120 on cores, could be reproduced by blocking cleavage at the MA-CA junction. This report and our data support a model in which a conformational change in gp41 occurs following cleavage of Gag by the viral protease, leading to a less stable interaction of gp41 and gp120. However, some isolates, perhaps most primary isolates and some lab-adapted strains, maintain a strong gp41/gp120 interaction even following cleavage of Gag. For the purpose of developing pseudovirions as a vaccine platform for Env presentation, it may be advantageous to use an immature core in order to avoid gp120 loss, and combining this with a primary isolate Env or stable T cell Env may produce the most stable particle.

A secondary goal of our study was to attempt to enhance the amount of Env present on pseudovirion particles through overexpression of gp160. Using codon-optimized gag and env genes, we achieved rapid and high-level expression. However, we were unable to achieve a significant decrease in Gag-to-Env ratio despite very efficient expression of gp160. Our Gag-to-Env molar ratios were remarkably similar to those of Chertova et al. (Chertova et al., 2002). In this study, a ratio of 60:1 was found for almost all viral isolates tested. These studies suggest that there may be constraints on the number of Env trimers on viral particles, and that the limiting factor may not be gp160 expression itself. Additional studies are needed to address the nature of these limitations. It may be desirable to utilize Env molecules with deletions of the cytoplasmic tail in order to enhance the amount of gp120 on the particle surface. We expect, however, that this would abrogate any benefit in stability achieved through the use of an uncleaved Gag core.

Overall, we believe that these results are encouraging for the development of pseudovirion-based vaccines. The use of an uncleaved Gag core and a primary isolate Env should result in the production of particles that do not shed gp120 at physiological temperatures, and that can present Env for induction of a strong humoral immune response. It is possible that these particles will also demonstrate enhanced gp120 retention in the presence of adjuvants. Experiments to address this hypothesis and to determine the immunogenicity of such pseudovirions are presently underway.

Materials and methods

Cell lines and plasmids

The human kidney cell line 293T was maintained in DMEM (Dulbecco’s modified Eagle medium, high glucose) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 2 mM penicillin-streptomycin. H9 leukemic T cells were obtained from Dr. Robert Gallo through the NIH AIDS Research and Reference Reagent Program and maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 2 mM penicillin-streptomycin. pNL4-3 is an expression plasmid encoding a complete infectious clone of HIV-1 (Adachi et al., 1986). pNLPr− is a proviral expression plasmid that contains a triple alanine substitution in the protease active site. This plasmid was created in the Aiken lab (Vanderbilt) by inserting a BssHII to EcoR1 fragment from R9.Pr− (Wyma et al., 2000) into pNL4-3. The construction of codon-optimized SF2 Pr55Gag expression plasmid pCMV Km2.GagMod.SF2 and SF2 gag-protease expression plasmid pCMV Km2.GagProtMod.SF2 (GP2) have been previously described (zur Megede et al., 2000). The envelope expression plasmid pCDNA 3.1(Zeo)-BaLgp160opt was produced by subcloning the codon-optimized Bal sequence from pMR1W1-9 (Fouts et al., 2000) into pCDNA 3.1(Zeo) (Invitrogen, San Diego, CA).

Virus production and purification

HIV-1 virions were produced as indicated in the Results section by transfection of proviral DNA in 293T cells, propagation of virus in H9 cells, or propagation of virus in PBMC blasts derived from normal donors. Biological stocks of HIV-1 isolate BaL were obtained from Drs. Suzanne Gartner, Mikulas Popovic, and Robert Gallo, MN from Robert Gallo, and SF162 from Dr. Jay Levy, all through the NIH AIDS Research and Reference Reagent Program. For transfections, 10 μg of proviral DNA was transfected into 293T cells on 10 cm2 dishes using the calcium phosphate/BBS transfection method (O’Mahoney and Adams, 1994). Typically 10 10 cm2 dishes of 293T cells were transfected at 30-40% confluence and incubated at 35 °C, 4% CO2 for 16 h. The cells were washed and fresh DMEM applied, and further incubation carried out at 37 °C and 5% CO2 for 48 h prior to harvesting. For labeling experiments, 75 μCi/ml of [35S]cysteine/methionine was applied in cysteine and methionine-deficient DMEM, and labeled viruses were harvested after 24 h. For production of laboratory-adapted HIV-1 isolates in H9 cells, 5 × 106 H9 cells were infected with 200 ng of a frozen biological stock of HIV strains NL4-3 or MN. Virus replication was allowed to proceed for 7 days, at which time syncytia formation in the culture was evident. H9 supernatants were then harvested, filtered through a 0.45 μm filter, and assessed for p24 and gp120 content. Primary isolates of HIV-1 were propagated in PHA blasts produced from PBMCs derived
from HIV-uninfected volunteer donors. In these experiments, 200 ng of primary isolate biological stock was incubated with 5 × 10⁶ PBMCs. PBMC cultures were maintained in RPMI 1640 with 15% FCS and 20 U/mL IL-2, and virus replication allowed to proceed for 7 days prior to harvesting. Supernatants were then filtered through a 0.45 μm filter. All virus preparations (from 293T, H9, or PBMC) were purified by ultracentrifugation through a 20% sucrose cushion (100,000 g for 3 h, 0 °C). Viral pellets were then resuspended in PBS with 0.5% BSA, and 1-mL aliquots were stored at −80 °C. Each aliquot of virus was thawed only once and used for a single experiment.

Pseudovirion production and purification

Pseudovirions were produced by cotransfection of 293T cells with codon-optimized Gag expression construct pCMV/Km2.GagMod.SF2 and codon-optimized Bal gp160 expression construct pcDNA3.1(Zeo)-Bal.Lgp160opt, or by cotransfection of codon-optimized Gag-Pro expression construct pCMV.Km2.GagProtMod.SF2 (GP2) and pcDNA3.1(Zeo)-Ba.Lgp160opt. Purification and analysis of pseudovirions was identical to that described for HIV-1 virions above.

Determination of gp120 concentration using a gp120 capture ELISA

The gp120 concentration of HIV-1 virions and pseudovirion preparations was measured by a capture ELISA similar to that of Moore and colleagues (Moore et al., 1992). The anti-gp120 capture antibody, D7324 (Cliniqa, Fallbrook, CA), was coated onto 96-well plates at a concentration of 4 μg/mL in PBS overnight at 37 °C. The plates were blocked with 5% fetal calf serum in PBS for 1 h at 37 °C. Viruses were diluted in Env sample diluent (10% FCS, 0.5% Triton X-100 in PBS) and incubated on D7324-coated plates for 2 h at 37 °C. The amount of captured gp120 was determined using pooled HIV positive patients’ sera at a dilution of 1:6000 in env sample diluent for 1 h at 37 °C. Detection was performed using a horseradish peroxidase-conjugated goat anti-human IgG (Pierce, Rockford, IL) at a dilution of 1:6000 in env sample diluent for 1 h at 37 °C. Colorimetric analysis was performed using the Immunopure TMB Substrate Kit (Pierce, Rockford, IL) and absorbance was read at 450 nm. Recombinant Bal gp120, obtained from the Division of AIDS, NIAID, through the NIH AIDS Research and Reference Reagent Program, was used for the standard curve. The assay was also calibrated using recombinant MN gp120 obtained through the same source. The linear detection of gp120 by this assay was sensitive to less than 10 pg of gp120.

HIV-1 envelope stability assay

Purified HIV-1 and pseudovirion preparations containing 10–20 ng of particle-associated gp120 were resuspended in 200 μL of PBS containing 0.5% BSA and were incubated in the presence of increasing amounts of sCD4 (Progenics Pharmaceuticals, Tarrytown, NY) at 0, 4, and 37 °C for 2 h. Virions were then purified by centrifugation through a 250 μl 20% sucrose cushion in a 1.5 ml microfuge tube at 100,000 g for 1.5 h at 0 °C using a Sorvall RP45A rotor. Purified virions or pseudovirions were then resuspended in Env sample diluent (10% fetal calf serum, 0.5% Triton X-100, in PBS) for analysis of gp120 content by previously described gp120 capture ELISA. Purified HIV-1 and pseudovirion preparations were also assayed for their stability to increasing temperatures. Aliquots of virus stocks containing 10–20 ng of particle-associated gp120 were resuspended in 200 μL of PBS containing 0.5% BSA and incubated at 0, 4, 22, 37, 50, and 65 °C for 2 h. Virions were then purified as previously described and gp120 content determined by the gp120 capture ELISA.

Determination of p24 concentration by ELISA

The p24 content of HIV-1 virions and pseudovirion preparations was performed using a p24 antigen capture ELISA. The murine anti-p24 capture antibody 183-H12-5C was obtained from Bruce Chesebro and Kathy Wehrly through the NIH AIDS Research and Reference Reagent Program. The capture antibody was coated onto 96-well plates at a dilution of 1:800 in PBS and incubated overnight at 37 °C. Plates were blocked for 1 h at 37 °C with 5% fetal calf serum in PBS. The detection of bound p24 was determined using HIV-Ig, obtained from NABI through the NIH AIDS Research and Reference Reagent Program, at a dilution of 1:20,000 for 1 h at 37 °C. Colorimetric analysis was performed using the Immunopure TMB Substrate Kit (Pierce, Rockford, IL) and absorbance was read at 450 nm. Recombinant p24 was used for the standard curve and sensitive to less than 20 pg of p24.

Gradient analysis of virions and pseudovirions

HIV-1 virions or pseudovirions were analyzed by centrifugation on linear 20–60% sucrose gradients, NL4-3 and NLPPr− virions were produced by transfecting 10 μg of proviral DNA into 293T cells on 10 cm² dishes using the calcium phosphate/BBS transfection method. Pseudovirions were produced by cotransfecting 4 μg of the codon-optimized Pr55Gag or Gag-Pro expression construct along with 4 μg of the codon-optimized Bal gp160 expression construct into 293T cells on 10 cm² dishes using the calcium phosphate/BBS transfection method. Typically three 10 cm² dishes of 293T cells were transfected at 30–40% confluence and incubated at 35 °C, 4% CO₂ for 16 h. The cells were then washed and fresh DMEM applied. Virus and pseudovirion preparations were metabolically labeled using 75 μCi/ml [³⁵S]cysteine/methionine applied in cysteine- and methionine-deficient DMEM. Labeled viruses and pseudovirions were harvested after 24 h, clarified by low-
speed centrifugation, filtered through a 0.45 μm filter, and then purified by ultracentrifugation through a 20% sucrose cushion (100,000 g for 3 h, 0 °C). Viral pellets were then resuspended in 1 mL of PBS with 0.5% BSA, and overlaid on linear 20–60% sucrose gradients. Ultracentrifugation was performed at 100,000 g overnight at 0 °C in a Beckman SW41 rotor. Equal fractions were collected and the density of each fraction determined using a refractometer. Samples were subsequently diluted in PBS and disrupted using RIPA buffer (1% NP-40, 0.1% SDS in PBS) and immunoprecipitated using HIV positive patients’ sera. Analysis was performed by SDS-PAGE and autoradiography, and quantitation performed with the Cyclone Phosphorimager System (Packard Instrument Co., Meriden, CT).

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References


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