2013-11-14

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HIV-1 Nef Responsiveness Is Determined by Env Variable Regions Involved in Trimer Association and Correlates with Neutralization Sensitivity

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http://dx.doi.org/10.1016/j.celrep.2013.09.028

SUMMARY

HIV-1 Nef and the unrelated murine leukemia virus glycoGag similarly enhance the infectivity of HIV-1 virions. We now show that the effects of Nef and glycoGag are similarly determined by variable regions of HIV-1 gp120 that control Env trimer association and neutralization sensitivity. Whereas neutralization-sensitive X4-tropic Env proteins conferred high responsiveness to Nef and glycoGag, particles bearing neutralization-resistant R5-tropic Envs were considerably less affected. The profoundly different Nef/glycoGag responsiveness of a neutralization-resistant and a neutralization-sensitive R5-tropic Env could be switched by exchanging their gp120 V1/V2 regions, which also switches their neutralization sensitivity. Within V1/V2, the same determinants governed Nef/glycoGag responsiveness and neutralization sensitivity, indicating that these phenotypes are mechanistically linked. The V1/V2 and V3 regions, which form an apical trimer-association domain, together determined the Nef and glycoGag responsiveness of an X4-tropic Env. Our results suggest that Nef and glycoGag counteract the inactivation of Env spikes with relatively unstable apical trimer-association domains.

INTRODUCTION

Nef is a small myristylated protein encoded by HIV-1 and other primate lentiviruses that constitutes a crucial virulence factor. Although not required for virus replication in cell culture, Nef is critical for high virus loads and for the development of AIDS in rhesus macaques infected with a pathogenic simian immunodeficiency virus (SIV) (Kestler et al., 1991). In humans infected with HIV-1, defects in *nef* have been associated with long-term nonprogression (Deacon et al., 1995; Kirchhoff et al., 1995). Nef downmodulates CD4 from the surface of infected cells (Aiken et al., 1994; Garcia and Miller, 1991; Mariani and Skowronski, 1993) and also downregulates major histocompatibility complex class I (MHC class I) molecules to protect infected cells from cytotoxic T cells (Cohen et al., 1999; Collins et al., 1998; Schwartz et al., 1996; Yang et al., 2002). Furthermore, Nef modulates T cell signaling (Abraham and Fackler, 2012; Baur et al., 1994; Du et al., 1995; Schindler et al., 2006) and inhibits T cell migration (Stolp et al., 2009). SIV Nef proteins also antagonize the restriction factor BST2 (Jia et al., 2009; Zhang et al., 2009).

Nef also enhances the intrinsic infectivity of progeny virions by a mechanism that remains poorly understood (Aiken and Trono, 1995; Chowers et al., 1994). It has been shown that high levels of cell surface CD4 sequester HIV-1 Env, and that Nef can counteract this effect by downregulating CD4 (Lama et al., 1999). However, the CD4 downregulation and infectivity enhancement functions of Nef can be dissociated (Goldsmith et al., 1995). Furthermore, Nef enhances HIV-1 infectivity in cells that lack CD4 or express a mutant CD4 that cannot be downregulated (Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al., 1995).

HIV-1 virions produced in the absence of Nef are defective at an early step of the replication cycle (Aiken and Trono, 1995; Miller et al., 1995; Schwartz et al., 1995). Nef is incorporated into virions in small quantities (Pandori et al., 1996; Welker et al., 1996), but its presence in HIV-1 particles is not sufficient to increase their infectivity (Laguette et al., 2009). Nef does not affect viral particle production, the processing of virion-associated Gag or Gag-pol products, or the structure or stability of the mature virion core (Forshey and Aiken, 2003; Miller et al., 1995). Although some reports suggest that Nef enhances the incorporation of Env (Day et al., 2004; Schiavoni et al., 2004), no effect on Env incorporation was seen in other studies (Miller et al., 1995; Pizzato et al., 2007). Although Nef may not enhance the initial phase of virus-cell fusion (Cavrois et al., 2004; Tobiume et al., 2003), Nef enhances the delivery of viral capsids into the cytosol (Campbell et al., 2004; Schaeffer et al., 2001). An effect of Nef on virus penetration is consistent with the observation that pseudotyping with pH-dependent Env proteins bypasses the requirement for Nef (Aiken, 1997; Luo et al., 1998). Interestingly, Nef decreases the sensitivity of HIV-1 to broadly

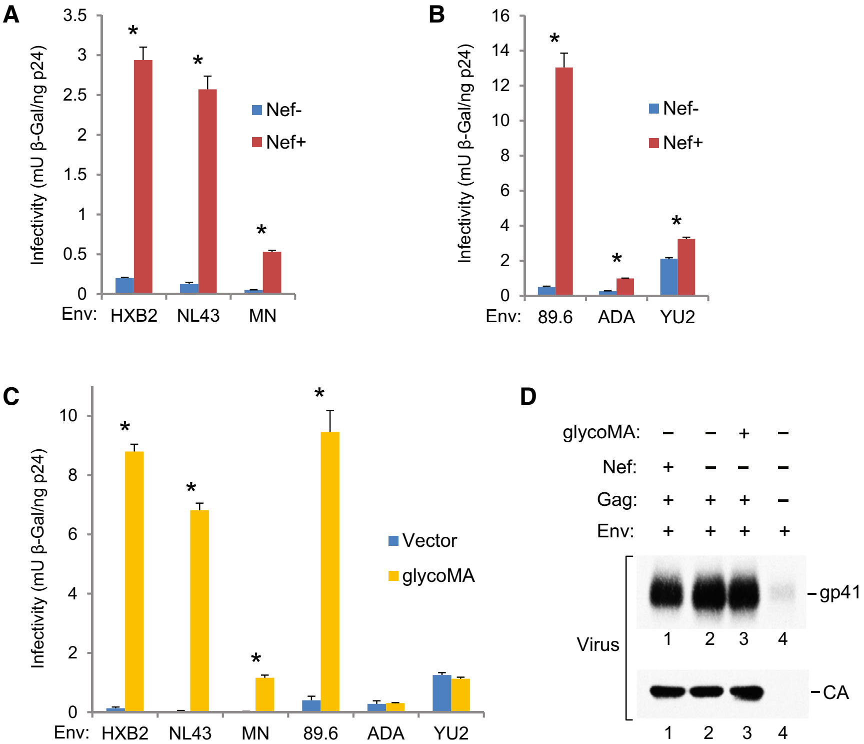

Figure 1. Env Determines HIV-1 Responsiveness to Nef and glycoGag

(A) The Env proteins of laboratory-adapted HIV-1 strains confer high responsiveness to Nef_{NL43}. Env^{-}/Nef^{-} and Env^{-}/Nef^{+} HIV-1_{NL43} particles *trans*-complemented with the indicated Env proteins were produced in Jurkat TAg cells, and infectivities normalized for p24 antigen were analyzed on TZM-bl indicator target cells.

(B) The Env proteins of primary HIV-1 isolates confer profoundly different degrees of responsiveness to Nef_{NL43}.

(C) Responsiveness to Nef correlates with responsiveness to glycoGag. Env^{-}/Nef^{-} HIV-1_{NL43} particles *trans*-complemented with the indicated Env proteins were produced in Jurkat TAg cells cotransfected with a small amount (30 ng) of a vector expressing glycoMA. Bars indicate the means of triplicate determinations in a single experiment. Error bars indicate ± SD. *$p < 0.005$ by two-tailed paired t test.

(D) Western blot shows that Nef and glycoMA do not affect the virion association of gp41. Purified recombinant viral particles produced in Jurkat TAg cells by full-length Env-deficient proviruses *trans*-complemented with Env_{HXB2} were analyzed. In lane 4, a Gag-deficient full-length provirus was used.

See also Figure S1.

neutralizing antibodies that target a specific region of gp41 (Lai et al., 2011).

Recently, it emerged that the unrelated glycosylated Gag (glycoGag) protein of Moloney murine leukemia virus (MLV) has a comparable effect on HIV-1 infectivity as Nef (Pizzato, 2010). MLV glycoGag is an alternative Gag molecule with an N-terminal extension that provides a transmembrane domain and causes its insertion into the plasma membrane (Pillemer et al., 1986). The Nef-like activity of glycoGag depends on its cytosolic N terminus, whereas the extracellular Gag portion is not strictly required (Pizzato, 2010). The effects of Nef and glycoGag on HIV-1 infectivity exhibit many similarities; for instance, they are similarly determined by the producer cell type, and the effects of both proteins are particularly pronounced in T lymphoid cells (Pizzato, 2010). Furthermore, in an analysis of particles pseudotyped with non-HIV Env proteins, the activities of Nef and glycoGag were similarly determined by Env (Pizzato, 2010).

We now show that even the responsiveness of different HIV-1 Env proteins to Nef varies dramatically and correlates strictly with their responsiveness to glycoGag. We find that responsiveness to Nef and glycoGag is similarly determined by variable regions of gp120 recently reported to form a trimer-association domain at the apex of the HIV-1 Env spike. We have also observed a close correlation between Nef/glycoGag responsiveness and sensitivity to neutralization by an antibody against the crown of the V3 loop. Together, our findings suggest that the effects of Nef and glycoGag on HIV-1 infectivity are determined by the quaternary conformation of the apex of the Env trimer.

RESULTS

HIV-1 Env Alleles Differ Significantly in Their Responsiveness to Nef

The enhancement of HIV-1 infectivity by Nef is viral isolate dependent (Luo and Garcia, 1996). Because Env is particularly variable, we investigated whether Env determines the magnitude of infectivity enhancement by Nef. Viral stocks were produced in Jurkat cells transfected with *env*-defective HIV-1_{NL43} proviruses that harbored either the wild-type (WT) NL43 *nef* gene or a frameshifted version, along with expression vectors for various HIV-1 Env alleles. The infectivities of the virus stocks, normalized for p24 content, were compared on TZM-bl target cells.

Nef_{NL43} enhanced the infectivity of HIV-1_{NL43} particles complemented with Env_{NL43} by approximately 20-fold (Figure 1A). Similarly, the infectivity of HIV-1_{NL43} particles pseudotyped with Env_{HXB2} was 10- to 20-fold higher in the presence of the Nef proteins of HIV-1_{NL43} or of the primary isolates 97ZA012 and 93BR020 (Figure 1A; Figures S1A and S1B). HIV-1_{NL43} and HIV-1_{HXB2} are both laboratory-adapted X4-tropic viruses, and their Env proteins are 97% identical. However, the infectivity of HIV-1_{NL43} particles pseudotyped with the more divergent Env protein of the laboratory-adapted X4-tropic HIV-1_{MN} strain was also enhanced more than 10-fold by Nef_{NL43} (Figure 1A).

We next examined the effects of Nef on the infectivities of HIV-1_{NL43} particles pseudotyped with primary Envs. The infectivity of HIV-1 particles bearing the R5X4-tropic $Env_{89.6}$ was strongly enhanced by Nef_{NL43} (more than 26-fold), whereas the infectivity of particles bearing the R5-tropic Env_{ADA} was only moderately affected (less than 4-fold; Figure 1B). Nef_{NL43}, $Nef_{97ZA012}$, and $Nef_{93BR020}$ had even smaller effects on the infectivity of particles complemented with the R5-tropic Env_{YU2} (Figures 1B, S1A, and S1B). Together, these results indicate that at least some R5-tropic primary Envs are considerably less responsive to Nef than the Env proteins of laboratory-adapted X4-tropic strains.

Responsiveness to Nef Correlates with Responsiveness to glycoGag

MLV glycoGag rescues the infectivity of *nef*-deficient HIV-1_{NL43} (Pizzato, 2010). To examine whether HIV-1 Env proteins differ in their responsiveness to glycoGag, we used a fully active N-terminal portion of glycoGag (Pizzato, 2010), here termed glycoMA. The responsiveness of HIV-1 Env proteins to glycoMA, measured on TZM-bl indicator cells, correlated closely with their responsiveness to Nef (Figure 1C). Even a small amount of the glycoMA expression vector (30 ng) had dramatic effects on the infectivities of *env*- and *nef*-deficient HIV-1_{NL43} particles *trans*-complemented with Env_{HXB2}, Env_{NL43}, or Env_{MN} (between 65- and 296-fold). Thus, the effects of glycoMA on particles bearing the X4-tropic Envs were even more pronounced than those of Nef. Neither glycoMA nor Nef affected viral particle production or Env incorporation (Figures 1D and S1C), consistent with earlier reports (Pizzato, 2010; Pizzato et al., 2007).

The glycoMA construct also strongly enhanced the infectivity of *env*- and *nef*-deficient HIV-1_{NL43} particles complemented with the R5X4-tropic $Env_{89.6}$ (Figure 1C), which was similarly responsive to Nef (Figure 1B). In marked contrast, glycoMA had little or no effect if the R5-tropic Env proteins of the primary ADA and YU2 isolates were used (Figure 1C), which also responded poorly to Nef (Figure 1B). Nef and glycoMA also considerably enhanced the infectivity of particles bearing $Env_{89.6}$, but not of particles bearing Env_{YU2}, for primary monocyte-derived macrophages (MDMs) (Figure S1D). Of note, the YU2 Env protein used in this study had a cytoplasmic domain that was mostly derived from HIV-1_{HXB2}. Taken together, our data thus suggested that responsiveness to Nef and glycoMA is determined by the ectodomain of HIV-1 Env. Consistent with this notion, Env_{HXB2} remained responsive to Nef in the absence of a cytoplasmic domain (Figure S1E).

Responsiveness to Nef and glycoGag Is Not Determined by Coreceptor Usage

Our results raised the possibility that coreceptor usage plays a role in the responsiveness of HIV-1 Env proteins to Nef and glycoGag. An alternative possibility was that there is a relationship between Nef/glycoGag responsiveness and general neutralization sensitivity. In support of this notion, all the X4-tropic laboratory-adapted Envs that were highly responsive to Nef and especially to glycoGag are highly susceptible to neutralization (Mascola et al., 1996). In contrast, the R5-tropic primary Envs that responded poorly to Nef and not at all to glycoGag are resistant to neutralization (Kolchinsky et al., 2001; Krachmarov et al., 2006).

We therefore examined the effects of Nef and glycoMA on the infectivities of particles bearing the Env proteins of the related primary isolates SF162 and JRFL, which are both R5-tropic but differ greatly in their sensitivity to neutralization (Pinter et al., 2004). Particles bearing these Env proteins had comparable baseline infectivities in the absence of Nef or glycoMA (Figure 2A). However, only the infectivity of particles bearing the neutralization-sensitive SF162 Env was substantially enhanced by Nef (11-fold) and glycoMA (34-fold). In marked contrast, Nef enhanced the infectivity of particles bearing the neutralization-resistant JRFL Env only about 2-fold, and glycoMA did not enhance their infectivity at all (Figure 2A). These results supported the notion that responsiveness to Nef and glycoGag is related to neutralization sensitivity. Furthermore, they excluded the possibility that responsiveness to Nef or glycoGag is determined by coreceptor usage because the SF162 and JRFL Env proteins both exclusively use CCR5 (Michael et al., 1998).

The Different Responsiveness of Two R5-Tropic Envs to Nef and glycoGag Is Determined by V1/V2

The inherent resistance of Env_{JRFL} to neutralization is largely determined by variable regions 1 and 2 (V1/V2) of gp120 (Pinter et al., 2004). These authors found that Env_{JRFL} becomes considerably more neutralization sensitive upon replacement of its V1/V2 region by that of neutralization-sensitive Env_{SF162} (Pinter et al., 2004). Conversely, Env_{SF162} becomes considerably more resistant to neutralization upon replacement of its V1/V2 region by that of Env_{JRFL} (Pinter et al., 2004). We therefore precisely exchanged the V1/V2 regions of the SF162 and JRFL Env proteins and examined the responsiveness of the resulting SF(JR V1/V2) and JR(SF V1/V2) chimeras to Nef and glycoMA.

The responsiveness of the SF(JR V1/V2) chimera to Nef was considerably reduced compared to that of the parental Env_{SF162} (2.4-fold versus 11-fold), and its responsiveness to glycoMA was dramatically lower (1.8-fold versus 39-fold) (Figure 2B). Conversely, the responsiveness of Env_{JRFL} to Nef was markedly increased by replacing its V1 and V2 regions with those of ENV_{SF162} (from 2.2-fold to 15-fold), and the increase in responsiveness to glycoMA was even more pronounced (from 1.2-fold to 15-fold) (Figure 2C). Taken together with previously published results on the neutralization sensitivity of the chimeras (Pinter et al., 2004), these observations implied that neutralization sensitivity and responsiveness to Nef or glycoMA can be switched simultaneously.

Exchange of V2 Region Is Sufficient to Switch Responsiveness of SF162 and JRFL Env to Nef and glycoGag

The V1/V2 region comprises a single topological entity that folds as four antiparallel β strands (McLellan et al., 2011). Strands A and B flank the highly variable V1 loop. Strands C and D, both located entirely within the V2 region, are connected by the less variable V2 loop (McLellan et al., 2011).

To examine the individual contribution of the V1 and V2 regions to the Nef/glycoGag responsiveness of Env_{SF162} and Env_{JRFL}, we generated chimeric Envs in which only the V1 or the V2 region was exchanged (Figure 3). The JR(SF V1) chimeric Env remained as poorly responsive to Nef as Env_{JRFL} and also remained entirely unresponsive to glycoMA (Figure 3A).

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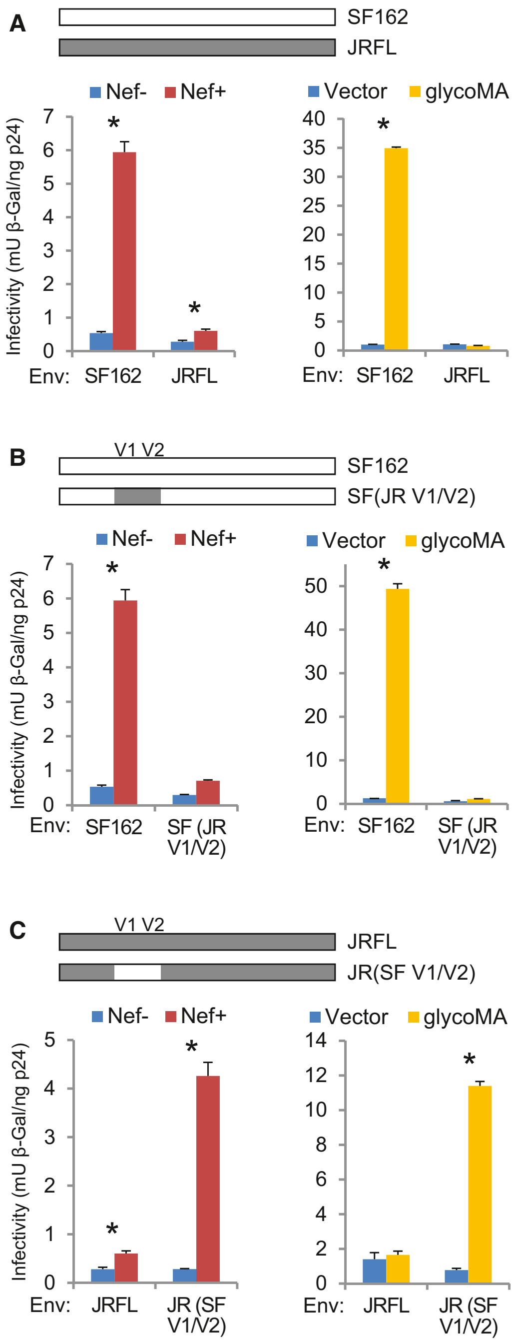

Figure 2. The Differential Nef/glycoGag Responsiveness of Two Related X5-Tropic Envs Is Determined by V1/V2

(A) Env_{SF162} confers high responsiveness to both Nef and glycoMA, whereas Env_{JRFL} confers poor responsiveness to Nef and resistance to glycoMA.

(B) Env_{SF162} becomes poorly responsive to Nef and resistant to glycoMA upon replacement of its V1/V2 region by that of Env_{JRFL}.

(C) Env_{JRFL} becomes highly responsive to Nef and glycoMA upon replacement of its V1/V2 region by that of Env_{SF162}. Bars indicate the means of triplicate determinations in a single experiment. Error bars indicate ± SD. *$p < 0.005$.

In striking contrast, the JR(SF V2) chimeric Env was nearly as highly responsive to both Nef and glycoMA as Env_{SF162} (Figure 3A).

We also examined the sensitivity of the JR(SF V2) chimera to cold inactivation, which is determined by the major variable regions of Env (Medjahed et al., 2013). While Env_{JRFL} was resistant to cold, the JR(SF V2) chimera was moderately sensitive. However, the level of sensitivity was not significantly influenced by Nef or glycoMA (Figure S2).

Next, we generated the reciprocal Env chimera in which the V2 region of the highly Nef/glycoGag-responsive Env_{SF162} was precisely replaced by that of the poorly responsive Env_{JRFL}. Despite being identical to Env_{SF162} except for V2, the SF(JR V2) chimeric Env closely resembled Env_{JRFL} in its poor response to Nef and unresponsiveness to glycoMA (Figure 3B). These results establish that the V2 region determines the different responsiveness of Env_{SF162} and Env_{JRFL} to Nef and glycoGag, whereas the V1 region plays no significant role.

The B-C Hairpin in V2 Governs the Nef and glycoGag Responsiveness of JRFL Env

Within the V2 region, Env_{SF162} and Env_{JRFL} differ at eight positions (Figure 4A). To examine which of these differences determine responsiveness to Nef and/or glycoGag, we generated Env_{JRFL} mutants that harbor either strand B, strand C, or the V2 loop together with strand D of Env_{SF162}, yielding the JR(SF B strand), JR(SF C strand), and JR(SF V2/D strand) Envs, respectively. None of these mutants was significantly more responsive to Nef or glycoMA than the poorly responsive parental Env_{JRFL} (Figure 4B). We also examined a versions of Env_{JRFL} that harbored both strand B and strand C of Env_{SF162}, denoted JR(SF B-C hairpin), and a version of the JR(SF C strand) chimera with an N160K mutation in strand B, denoted JR/N160K(SF C strand). The N160K mutation eliminates an N-linked glycosylation site that is present in Env_{JRFL} but absent from Env_{SF162} (Pinter et al., 2004). The JR(SF B-C hairpin) and JR/N160K(SF C strand) chimeras exhibited robust responsiveness to Nef and glycoMA (Figures 4B and 4C). These results indicate that differences in the B-C hairpin largely account for the differences in responsiveness of Env_{SF162} and Env_{JRFL} to Nef and glycoGag.

Nef/glycoGag Responsiveness Correlates Closely with Sensitivity to Neutralization by a V3 Monoclonal Antibody

The neutralization phenotypes of Env_{SF162} and Env_{JRFL} could be switched by swapping their V1/V2 regions, which had particularly pronounced effects on sensitivity to neutralization by V3-specific monoclonal antibodies (mAbs) such as mAb 447-52D (Pinter et al., 2004). Because exchanging the V1/V2 regions of Env_{SF162} and Env_{JRFL} also switches their responsiveness to Nef and glycoGag, we further examined the relationship between Nef/glycoGag responsiveness and sensitivity to neutralization by mAb 447-52D.

Irrespective of whether Nef was present or absent during virus production, viral particles bearing Env_{JRFL} were resistant to 447-52D, whereas particles bearing the JR(SF V1/V2) Env chimera were potently neutralized (Figure 5A). Interestingly, the

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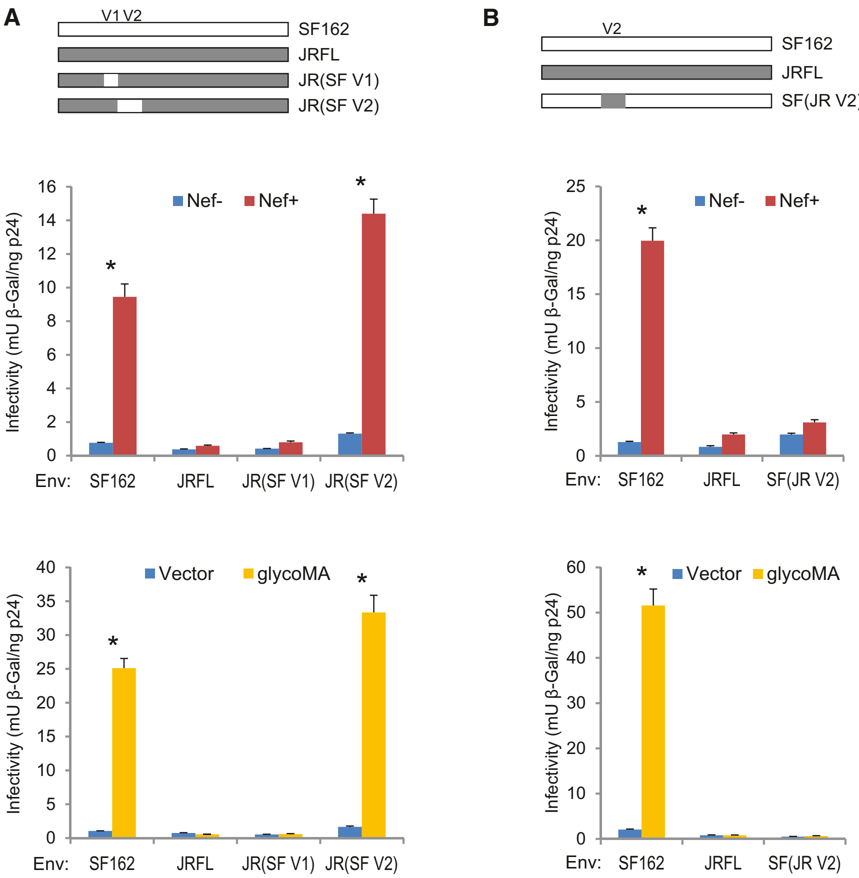

Figure 3. Replacement of V2 Region Is Sufficient to Switch Nef and glycoGag Responsiveness

(A) Env$_{JRFL}$ becomes as highly responsive to Nef and glycoMA as Env$_{SF162}$ upon replacement of its V2 but not of its V1 domain by that of Env$_{SF162}$.

(B) Env$_{SF162}$ becomes as poorly responsive to Nef and glycoMA as Env$_{JRFL}$ upon replacement of its V2 region by the corresponding JRFL sequence. Bars indicate the means of triplicate determinations in a single experiment. Error bars indicate ± SD. *$p < 0.005$.

See also Figure S2.

JR(SF V1) chimera was as resistant to 447-52D as the parental Env$_{JRFL}$ (Figure 5B), whereas the JR(SF V2) chimera was nearly as potently neutralized as the JR(SF V1/V2) chimera (Figure 5C). Thus, the SF162-derived V2 region conferred both Nef/glycoGag responsiveness and neutralization sensitivity, whereas the SF162-derived V1 region conferred neither.

As recently reported (Lai et al., 2011), Nef reduced the sensitivity of particles bearing Env$_{JRFL}$ to neutralization by mAb 2F5 (Figure S3), which targets the membrane-proximal external region (MPER) of gp41 (Purtscher et al., 1994). Nef also partially protected the JR(SF V1) chimera, which exhibited a comparable susceptibility to 2F5 as Env$_{JRFL}$ (Figure S3). However, Nef did not significantly protect the JR(SF V1/V2) and JR(SF V2) chimeras, which were more susceptible to 2F5 than was Env$_{JRFL}$ (Figure S3).

We also examined the effects of 447-52D on the JR(SF B strand) and JR(SF C strand) Env chimeras, which resemble the parental Env$_{JRFL}$ in their poor responsiveness to Nef and glycoGag. Both chimeras were nearly as resistant to 447-52D as the parental Env$_{JRFL}$ (Figure 6). In contrast, the Nef- and glycoGag-responsive JR(SF B-C hairpin) and JR/N160K(SF C strand) chimeras were clearly neutralized by 447-52D (Figure 6). Thus, determinants within the SF162-derived strands B and C together, but not the individual strands, simultaneously conferred significant Nef/glycoGag responsiveness and neutralization sensitivity.

V1/V2 and V3 Together Determine the Nef and glycoGag Responsiveness of a Laboratory-Adapted Env

Env$_{SF162}$ and Env$_{JRFL}$ are both R5-tropic primary isolates (Michael et al., 1998). To examine whether the V1/V2 region also governs the Nef and glycoGag responsiveness of a laboratory-adapted X4-tropic Env, we used a previously described version of the highly Nef- and glycoGag-responsive Env$_{HXB2}$ that has the V1/V2 region precisely replaced by that of the poorly Nef- and glycoGag-responsive Env$_{YU2}$ (Figure 7A) (Sullivan et al., 1998). Compared to the parental Env$_{HXB2}$, the HX(YU V1/V2) chimera exhibited no decreased responsiveness to Nef (Figure 7B) or glycoMA (Figure 7C). Thus, the transfer of the V1/V2 region by itself did not confer a YU2-like phenotype.

Whereas Env$_{SF162}$ and Env$_{JRFL}$ have similar V3 regions, Env$_{HXB2}$ and Env$_{YU2}$ differ substantially in V3. Therefore, we examined the phenotype of another previously described Env chimera (Sullivan et al., 1998), here called HX(YU V3), that is identical to Env$_{HXB2}$ except that the V3 region is from Env$_{YU2}$ (Figure 7A). In contrast to the parental Env$_{HXB2}$, the HX(YU V3) chimeric Env can use CCR5 as a coreceptor (Choe et al., 1996). In our hands, the presence of the YU2 V3 region in the Env$_{HXB2}$ background conferred a dramatically increased baseline infectivity for TZM-bl cells, which have high levels of CCR5 (Figures 7B and 7C). Although to a lesser degree than the parental Env$_{HXB2}$, the HX(YU V3) Env chimera clearly remained responsive to Nef (Figure 7B) and glycoMA (Figure 7C). These results suggested that the YU2 V3 region alone mitigated the effects of Nef and glycoMA, albeit to a limited extent.

A recent cryo-EM structure indicates that the V1/V2 and V3 regions of gp120 together form a trimer-association domain at the apex of the unliganded Env spike (Mao et al., 2012). Based on this model, we examined the possibility that the V1/V2 and V3 regions together determine the effects of Nef and glycoGag on virus infectivity. We found that a chimera designated HX(YU V1/V2/V3), which harbors both the YU2 V1/V2 and YU2 V3 regions in the Env$_{HXB2}$ background (Figure 7A), exhibited a

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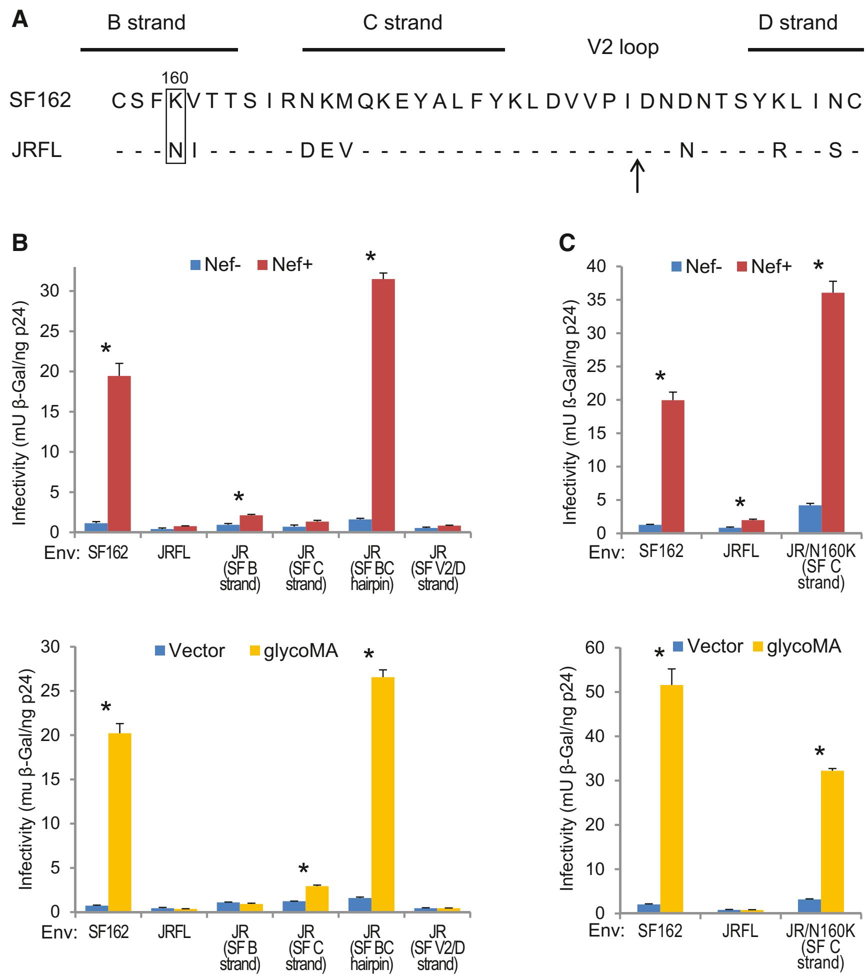

Figure 4. B-C Hairpin of V2 Region Governs Nef and glycoGag Responsiveness of Env_{JRFL}

(A) Alignment of the V2 regions of Env_{SF162} and Env_{JRFL} is shown. Identical residues are indicated by hyphens, and β strands by lines above the alignment (McLellan et al., 2011). The arrow indicates were sequences were joined to generate the JR(SF B–C hairpin) and JR (SF V2/D strand) chimeras.

(B) The entire B-C hairpin of Env_{SF162} confers an SF162-like Nef and glycoGag responsiveness to Env_{JRFL}, whereas individual strands from the SF162 V2 region do not.

(C) The N160K mutation together with strand C of Env_{162} confer Nef and glycoGag responsiveness to Env_{JRFL}. Error bars indicate ± SD. $^*p < 0.005$.

baseline infectivity that was only moderately higher than that of the parental Env_{HXB2}. However, in contrast to the highly responsive Env_{HXB2}, the HX(YU V1/V2/V3) chimera was poorly responsive to Nef (less than 2-fold; Figure 7B) and completely unresponsive to glycoMA (Figure 7C). Together, these results support the notion that Nef and glycoGag responsiveness are governed by the trimer-association domain composed of V1/V2 and V3.

DISCUSSION

This study shows that the effects of Nef and glycoGag on the infectivity of HIV-1 virions are determined by the V1/V2 and V3 regions of gp120. Recent cryo-EM structures indicate that these variable regions are near the apex of the Env trimer (Liu et al., 2008; White et al., 2010) and together form a trimer-association domain that stabilizes the unliganded Env spike (Mao et al., 2012). Our data suggest that Nef and glycoGag responsiveness is controlled by the architecture of this trimer-association domain, which has been proposed to be metastable (Mao et al., 2012). In support of this model, we have observed a close relationship between responsiveness to Nef or glycoGag and sensitivity to neutralization by a V3 mAb.

Our data show that the V1/V2 region of gp120 determines the markedly different Nef and glycoGag responsiveness of two related R5-tropic Envs that have similar V3 regions. The highly responsive Env_{SF162} became as unresponsive as Env_{JRFL} in the presence of the JRFL V1/V2 region. Conversely, Env_{JRFL} became highly responsive in the presence of the SF162 V1/V2 region. Interestingly, the V1/V2 region also determines the high neutralization sensitivity of Env_{SF162} and the neutralization resistance of Env_{JRFL} (Pinter et al., 2004). The differential sensitivities of these Env proteins to neutralization by antibodies targeting V3 or other sites on gp120 can be switched by exchanging their V1/V2 regions (Pinter et al., 2004), consistent with the notion that V1/V2 shields the V3 region and other functionally important sites from recognition by antibodies (Pantophlet and Burton, 2006).

The V1/V2 region is not strictly required for virus replication (Cao et al., 1997; Johnson et al., 2002; Saunders et al., 2005), but nevertheless plays an important role in holding together unliganded Env trimers (Hu et al., 2011; Liu et al., 2008). The V1 and V2 regions are proposed to contact each other at the apex of the trimer in the unliganded state and to move away upon CD4 binding (Liu et al., 2008). It was recently shown that the V1/V2 region, together with the V3 region, restrains the tendency of HIV-1 Env spikes to spontaneously assume the neutralization-sensitive CD4-bound conformation (Kwon et al., 2012). Consistent with this view, a recent cryo-EM structure of the unliganded Env_{JRFL} trimer indicates that V1/V2 together with V3 form an apical trimer-association domain that restricts antibody access to conserved regions within the interior of the cage-like structure (Mao et al., 2012). Accordingly, the globally increased neutralization sensitivity of Env_{JRFL} in the presence of the SF162 V1/V2 region suggests a propensity to assume a more open quaternary conformation.

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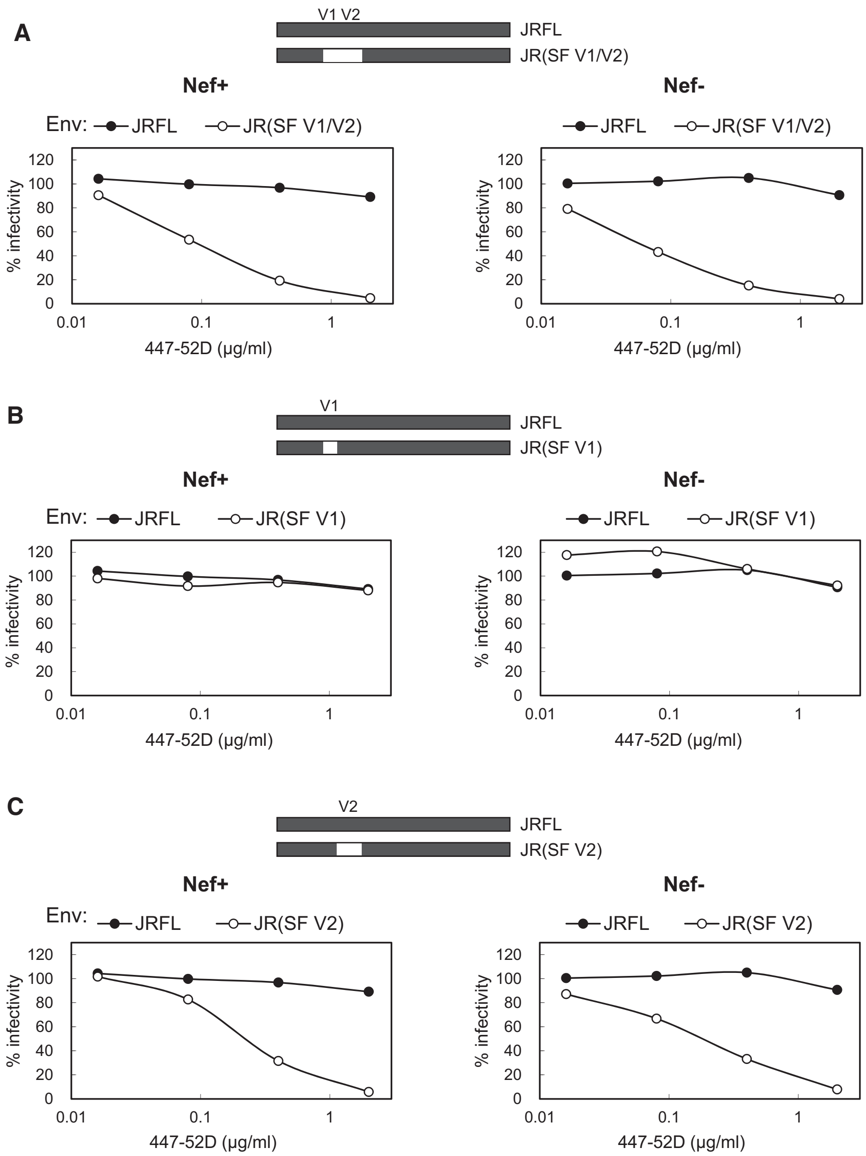

Figure 5. SF162 V2 Region Confers Sensitivity to Neutralization by a V3 mAb

(A) Neutralization curves confirming that the replacement of the V1/V2 region of Env_{JRFL} by that of Env_{SF162} confers sensitivity to neutralization by 447-52D are shown. Pseudotyped viruses were produced in the presence or absence of Nef, as indicated.

(B) Env_{JRFL} remains resistant to 447-52D in the presence of the Env_{SF162} V1 region.

(C) Replacement of the V2 region alone is sufficient to confer sensitivity to neutralization by 447-52D.

See also Figure S3.

There is evidence that within V1/V2 it is primarily the V2 region that confers protection against neutralization (O'Rourke et al., 2010, 2012; Stamatatos and Cheng-Mayer, 1998; Watkins et al., 2011). Similarly, we find that the transfer of the V2 region, but not of the V1 region, from Env_{SF162} to Env_{JRFL} is sufficient to confer sensitivity to neutralization by mAb 447-52, which recognizes an epitope at the tip of the V3 loop (Stanfield et al., 2004). The transfer of the V2 region also increased the sensitivity of Env_{JRFL} to the MPER mAb 2F5, particularly in the presence of Nef, which renders WT Env_{JRFL} resistant to 2F5 (Lai et al., 2011). Furthermore, the transfer of the V2 region conferred sensitivity to cold inactivation, which has been linked to the propensity to sample the CD4-bound conformation (Kassa et al., 2009). Interestingly, the transfer of the V2 region was also sufficient to confer an SF162-like responsiveness to Nef and glycoGag. Together, these findings suggest that Nef/glycoGag responsiveness is related to the propensity of Env to undergo conformational transitions, a property that has been called "intrinsic reactivity" (Haim et al., 2011).

The V2 region contains a conserved tripeptide reported to serve as a binding site for integrin $\alpha_4\beta_7$ (Arthos et al., 2008). However, a different region of V2 accounted for the differential Nef/glycoGag responsiveness of Env_{SF162} and Env_{JRFL}, namely, the tip of the B-C hairpin formed by the N terminus of V2. Modifications in the Env_{JRFL} B-C hairpin that conferred Nef/glycoGag responsiveness also conferred sensitivity to neutralization by 447-52D, supporting the notion that these phenotypes are related.

To transfer the Nef/glycoGag resistance of a primary R5-tropic Env to a laboratory-adapted X4-tropic Env with a highly divergent V3 region, it was necessary to replace both the V1/V2 and V3 regions of gp120. This result supports the model that Nef and glycoGag responsiveness reflect the configuration of the recently reported trimer-association domain at the membrane-distal apex of the spike (Mao et al., 2012). The cryo-EM structure of the unliganded Env_{JRFL} trimer indicates that the V1/V2 and V3 variable regions of this neutralization-resistant isolate together form a six-way junction at the gp120 interfaces (Mao et al., 2012). Thus, the simultaneous transfer of the V1/V2 and V3 regions of the highly neutralization-resistant Env_{YU2} to the neutralization-sensitive Env_{HXB2} may lead to a more stable six-way junction. It is noteworthy in this regard that the simultaneous transfer of the YU2 V1/V2 and V3 regions confers a higher degree of neutralization resistance to Env_{HXB2} than does the transfer of either region alone (Sullivan et al., 1998).

In one study, Nef and a sorting signal in the Env cytoplasmic tail each enhanced the efficiency of HIV-1 entry independently of the Env content of virions (Day et al., 2004). It has therefore been proposed that the sorting signal targets the trafficking of Env to a compartment optimal for the assembly of infectious virions, and that Nef modifies this compartment (Day et al.,

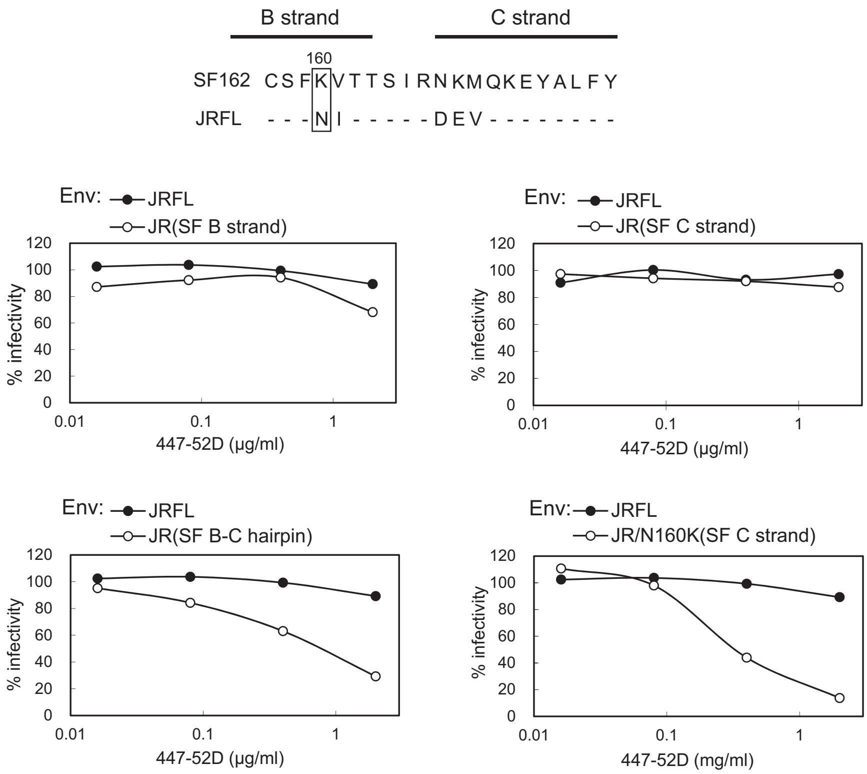

Figure 6. SF162 B-C Hairpin Confers Sensitivity to 447-52D

The neutralization sensitivity of particles pseudotyped with the indicated Env proteins in the presence of Nef was determined.

2004). It is conceivable that the fusogenicity of relatively unstable Env complexes benefits disproportionately from assembly in such a compartment. Interestingly, the enhancement of HIV-1 infectivity by Nef correlates with Nef-induced alterations of the endocytic recycling compartment (Madrid et al., 2005). These widespread alterations likely involve endosome-associated clathrin adaptor complexes (Madrid et al., 2005), providing a possible basis for the observation that the effect of Nef on HIV-1 infectivity depends on clathrin (Pizzato et al., 2007). Alternatively, Nef may affect the clathrin-mediated endocytosis or trafficking of a specific host factor that disproportionately influences the ability of relatively unstable Env trimers to maintain the high potential energy necessary for virus-cell fusion (Medjahed et al., 2013).

It has been reported that Nef does not affect the fusion of HIV-1 virions with target cells (Cavrois et al., 2004; Tobiume et al., 2003). However, in another study that employed a similar assay Nef-deficient viruses exhibited a 50% reduction in viral entry (Day et al., 2004). Although the defect in entry did not fully explain the much larger defect in infectivity, it was suggested that the highly concentrated inocula needed for the entry assay may have been responsible for the difference (Day et al., 2004). Our finding that gp120 variable regions determine the effect of Nef on HIV-1 infectivity supports the notion that Nef enhances entry.

Our results suggest that primary Envs generally do not possess the dramatic Nef responsiveness of laboratory-adapted Envs. However, primary Envs differ considerably in their intrinsic reactivities (Haim et al., 2011), and those relatively prone to conformational transitions upon stimulation may exhibit substantial Nef/glycoGag responsiveness.

EXPERIMENTAL PROCEDURES

Plasmids

The *env*-deficient HIV-1 proviruses HXB/Env$^-$/Nef$^+$ and HXB/Env$^-$/Nef$^-$ have been described (Dorfman et al., 2002), as have the vectors expressing $Nef_{97ZA012}$ and $Nef_{93BR020}$ (Pizzato et al., 2007). The vector expressing glycoMA (pBJ5-glycoMA) was made by inserting nt 360–926 of Moloney MLV (J02255) into pBJ5 after a Kozak sequence and an ATG initiation codon. The MLV sequence is followed by a sequence encoding an HA tag and a stop codon. Vectors expressing Env_{SF162} and Env_{JRFL} from pSVIIIenv have been described (Peters et al., 2004). V1/V2 chimeras were made by exchanging DraIII-StuI fragments between these vectors as described (Pinter et al., 2004). The V1/V2 chimeras were used to generate V1 and V2 chimeras by PCR-based cloning. Intra-V2 chimeras were made by PCR-based cloning or by site-directed mutagenesis. All these chimeras were expressed from the same vector (pSVIIIenv). The $pEnv_{HXB}$ and $pEnv_{HXB}\Delta CT$ plasmids are pBJ5-based expression vectors for WT and cytoplasmic tail-deleted Env_{HXB2} (Reil et al., 1998). A pBJ5-based expression vector for Env_{YU2} was generated by replacing the KpnI-XhoI fragment of $pEnv_{HXB}$ (nt 6347–8897 of HIV-1_{HXB2}) with the corresponding fragment from a pSVIIIenv-based Env_{YU2} expression vector (Sullivan et al., 1998). In the same manner, pBJ5-based expression vectors for Env_{HXB2} containing the YU2 V1/V2 region, the YU2 V3 region, or the YU2 V1/V2 region together with the YU2 V3 region were made by transferring KpnI-XhoI fragments from pSVIIIenv-based expression vectors (Sullivan et al., 1998) into $pEnv_{HXB}$.

Analysis of Virus Infectivity

Pseudovirions capable of a single round of replication were produced by transfecting Jurkat TAg cells with lipofectamine 2000 (Invitrogen). To examine the effects of Nef on HIV-1 infectivity, 1 µg of HXB/Env$^-$/Nef$^+$ or HXB/Env$^-$/Nef$^-$ was transfected along with 100 ng of a pSVIIIenv-based Env expression vector. To examine the effects of glycoGag, 1 µg HXB/Env$^-$/Nef$^-$ was transfected along with 100 ng of a pSVIIIenv-based Env expression vector and 30 ng of pBJ5-glycoMA or the empty vector. Additionally, an HIV-1 vector expressing GFP was cotransfected for macrophage infections. For the experiment shown in Figure 7, pBJ5-based Env expression vectors (0.5 µg) were used. Supernatants containing progeny virions were harvested 2 days post transfection, clarified by low-speed centrifugation, filtered through 0.45 µm pore filters, and then used immediately to infect TZM-bl indicator cells in triplicate in T25 flasks. Alternatively, primary human MDM prepared as described (Peters et al., 2004) were used as target cells because of their relative susceptibility to single-round infection with 89.6 and YU2 Env pseudovirions. Aliquots of the virus stocks were frozen for p24 antigen quantitation by a standard ELISA. Three days post infection, TZM-bl indicator cells were lysed in 400 µl reporter lysis buffer (Promega), and β-gal activity was measured according to the manufacturer's instructions. Infected MDM were quantified by counting GFP-positive cells in five random fields under a fluorescent microscope equipped with an X20 objective. Values were normalized for the amount of p24 antigen present in the supernatants used for infection.

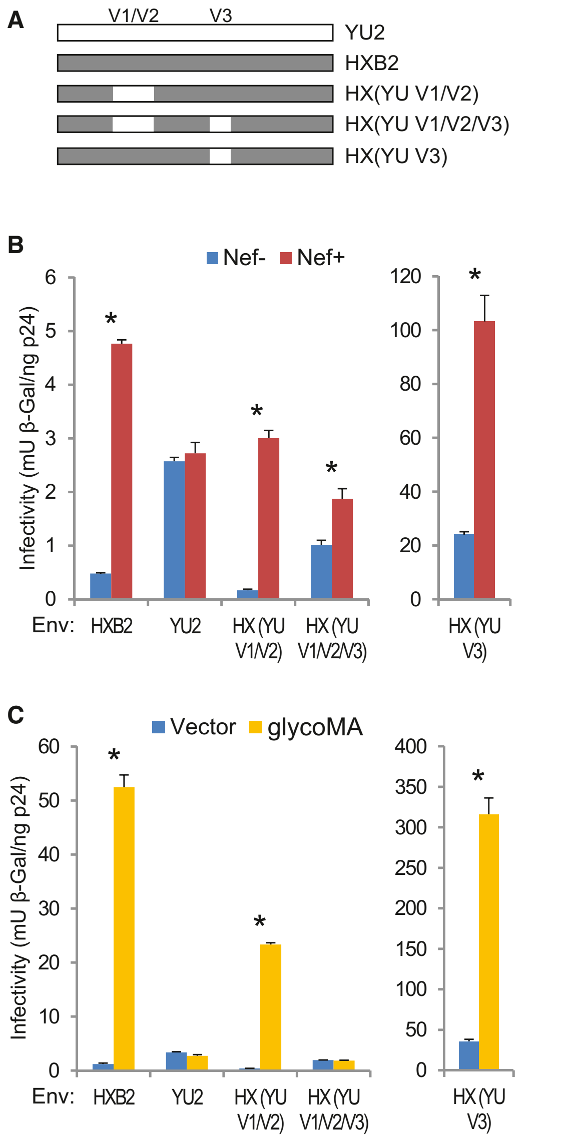

Figure 7. V1/V2 and V3 Regions Together Determine Nef and glycoGag Responsiveness of a Laboratory-Adapted Env
(A) Schematic illustration of the Env proteins examined is shown.
(B) Env_{HXB2} becomes poorly responsive to Nef only after the simultaneous replacement of its V1/V2 and V3 regions by those of Env_{YU2}.
(C) Responsiveness of the Env chimeras to glycoMA correlates with their responsiveness to Nef. Bars indicate the means of triplicate determinations in a single experiment. Error bars indicate ± SD. *$p < 0.005$.

Neutralization Assays

Pseudovirions produced in 293T cells transfected with $HXB/Env^{-}/Nef^{+}$ or $HXB/Env^{-}/Nef^{-}$ and a pSVIIIenv-based Env expression vector were quantified by p24 antigen ELISA. Pseudovirions equivalent to 60 ng p24 were then incubated in a total volume of 200 μl in the presence of mAb 447-52D at 0.016, 0.08, 0.4, and 2 μg/ml or of mAb 2F5 at 0.24, 1.2, 6, and 30 μg/ml. After 1 hr of incubation at 37°C, the mixture was added to TZM-bl indicator cells that had been seeded into T25 flasks the day before. Three days later, the indicator cells were lysed and analyzed for β-gal activity.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.09.028.

ACKNOWLEDGMENTS

We thank Paul Clapham for mAb 447-52D and Env expression vectors. We also thank Joseph Sodroski for pHIvec2.GFP and Env expression vectors. Chessie 8, 2F5, and 183-H12-5C were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. George Lewis, Hermann Katinger, and Bruce Chesebro, respectively. This work was supported by NIH grant number AI077412 and the UMass CFAR (P30 AI042845).

Received: May 10, 2013
Revised: August 19, 2013
Accepted: September 20, 2013
Published Date: October 24, 2013

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