Envelope Glycoprotein Internalization Protects Human and Simian Immunodeficiency Virus-Infected Cells from Antibody-Dependent Cell-Mediated Cytotoxicity

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ABSTRACT

The cytoplasmic tails of human and simian immunodeficiency virus (HIV and SIV, respectively) envelope glycoproteins contain a highly conserved, membrane-proximal endocytosis motif that prevents the accumulation of Env on the surface of infected cells prior to virus assembly. Using an assay designed to measure the killing of virus-infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC), we show that substitutions in this motif increase the susceptibility of HIV-1- and SIV-infected cells to ADCC in a manner that directly correlates with elevated Env levels on the surface of virus-infected cells. In the case of HIV-1, this effect is additive with a deletion in vpu recently shown to enhance the susceptibility of HIV-1-infected cells to ADCC as a result of tetherin-mediated retention of budding virions on the cell surface. These results reveal a previously unappreciated role for the membrane-proximal endocytosis motif of gp41 in protecting HIV-1- and SIV-infected cells from antibody responses by regulating the amount of Env present on the cell surface.

IMPORTANCE

This study reveals an unappreciated role for the membrane-proximal endocytosis motif of gp41 in protecting HIV-1- and SIV-infected cells from elimination by Env-specific antibodies. Thus, strategies designed to interfere with this mechanism of Env internalization may improve the efficacy of antibody-based vaccines and antiretroviral therapies designed to enhance the immunological control of HIV-1 replication in chronically infected individuals.

Enterviral envelope glycoproteins, including those of the human and simian immunodeficiency viruses (HIV and SIV, respectively), have unusually long cytoplasmic domains compared to those of other retroviruses. Although the function of this domain is not fully understood, it is known to contain sequences important for regulating Env trafficking in HIV-1- and SIV-infected cells (1–5). Perhaps the best characterized of these is a highly conserved binding site for the clathrin adapter protein 2 (AP-2) in the membrane-proximal region of the gp41 cytoplasmic domain (CD) (6, 7). Amino acid substitutions in this tyrosine-based motif (YXXΦ, where Φ represents any hydrophobic residue and X represents any residue) increase Env expression on the surface of infected cells and Env incorporation into virions (1, 7–9). This motif is also required for optimal HIV-1 infectivity (10) and for SIV pathogenesis in macaques (11).

We hypothesized that by regulating steady-state Env levels on the cell surface prior to the assembly and release of infectious virus, gp41 CD-dependent endocytosis may reduce the susceptibility of infected cells to Env-specific antibodies. Previous studies have shown that Vpu-mediated downregulation of tetherin and Nef-mediated downregulation of CD4 protect HIV-1-infected cells from antibody-dependent cell-mediated cytotoxicity (ADCC) by limiting Env exposure on the cell surface (12–15). Here, we show increased susceptibility to ADCC in cells infected with HIV-1 and SIV mutants carrying substitutions that disrupt the membrane-proximal AP-2 binding site in the gp41 tail. Greater susceptibility to ADCC correlates with higher levels of Env on the cell surface, indicating that endocytosis of Env may be another mechanism by which virus-infected cells evade the antibody responses of their hosts.

MATERIALS AND METHODS

Production of mutant viruses. Amino acid substitutions were introduced at key positions of possible trafficking motifs in the gp41 CDs of SIVmac239 (Fig. 1A) as well as of HIV-1NL4-3, HIV-1NL4-3Δvpu, and HIV-1JR-CSF (Fig. 1B). Changes were introduced into infectious molecular clones by site-directed mutagenesis, while maintaining the sequences of overlapping open reading frames. As a safety precaution for producing vesicular stomatitis virus G protein (VSV-G) pseudotyped virus, a 2-bp deletion in vif was introduced in HIV-1JR-CSF, resulting in a premature stop codon followed by a frameshift. After sequence confirmation, plasmids were transfected into HEK293T cells, and virus stocks were produced by harvesting cell culture supernatant at 48 and 72 h posttransfection. Since HIV-1JR-CSF showed low infectivity, this virus was pseudotyped with VSV-G. Virus concentrations were determined by anti-p24 or anti-p27 enzyme-linked immunosorbent assay (ELISA). Molecular clones were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, as follows: SIVmac239 SpX from Ronald C. Desrosiers, pNL4-3 from Malcolm Martin, and pYK-JRCSF from Irvin

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Chen and Yoshio Koyanagi. The construction of pNL4-3 Δp7vpu was previously described (16).

**ADCC assay.** ADCC activity was measured as previously described (17, 18). CEM.NKR-CCR5-sLTR-Luc cells, which express luciferase (Luc) under the control of a Tat-inducible promoter, were infected by spinoculation in the presence of 40 μg/ml Polybrene. At 4 days postinfection, target cells were incubated with an NK cell line stably expressing either human or rhesus macaque CD16 in the presence of purified IgG from HIV-positive donors (HIVIG), plasma from an SIV-infected rhesus macaque, or eCD4-Ig (Invitrogen) was used to exclude nonviable cells, and staining for Env was performed using either HIVIG, plasma from an SIV-infected macaque, or eCD4-Ig mimetic sulfopeptide (19, 20). After an 8-h incubation, luciferase activity was measured. NK cells cultured with either uninfected or infected target cells in the absence of antibody or plasma were used to determine maximal and background luciferase activity, respectively. Antibody concentrations for half-maximal killing (50% ADCC) and values for the area under the ADCC curve (AUC) were calculated from percent relative light units (RLU), as previously described (17).

**Flow cytometry.** Envelope staining on the surface of infected cells was performed using an established protocol (12). Three days postinfection, target cells were surface stained for CD45 (peridinin chlorophyll protein [PerCP]; clone 2D1), CD4 (Alexa Fluor 700; clone RPA-T4), and intracellular Gag (fluorescein isothiocyanate [FITC]) (for HIV-1, clone FH190-1-1; for SIV, clone 55-2F12). Live/Dead fixable dead cell aqua stain (Invitrogen) was used to exclude nonviable cells, and staining for Env was performed using either HIVIG, plasma from an SIV-infected rhesus macaque, or eCD4-Ig mim22, a CD4-Ig fusion with a CCR5-mimetic sulfopeptide (19, 20). After an 8-h incubation, luciferase activity was measured. NK cells cultured with either uninfected or infected target cells in the absence of antibody or plasma were used to determine maximal and background luciferase activity, respectively. Antibody concentrations for half-maximal killing (50% ADCC) and values for the area under the ADCC curve (AUC) were calculated from percent relative light units (RLU), as previously described (17).

**Statistical analysis.** Fifty percent ADCC titers, AUC values, and gMFIs for Env staining were compared by one-way analysis of variance (ANOVA) with a Holm-Sidak correction for multiple comparisons. For gMFIs for Env staining were compared by one-way analysis of variance (ANOVA) with a Holm-Sidak correction for multiple comparisons. For each group, the antibody concentration for half-maximal killing by more than 2 orders of magnitude was determined by the 50% ADCC titer (Fig. 2F) (P < 0.0001) and resulting in more than a 4-fold difference in area under the ADCC curve (AUC) values (Fig. 2C) (P < 0.0001).

In accordance with the ADCC measurements, Env levels on the surface of virus-infected cells were higher for the Y721G mutant (Fig. 2D). Whereas the geometric mean fluorescence intensity (gMFI) of Env staining was 3.9-fold higher for the Y721G mutant (Fig. 2E) (P = 0.0006), there was no detectable difference in Env staining levels for the Y768F and Y795F mutants (Fig. 2E). Surface expression of Env also correlated with susceptibility to ADCC as measured by the 50% ADCC titer (Fig. 2F) (P < 0.0001) and AUC values (Fig. 2G) (P = 0.0005).

**Disruption of the HIV-1 gp41 AP-2 binding site enhances the susceptibility of infected cells to ADCC.** A corresponding tyrosine-to-glycine substitution at amino acid position 710 of HIV-1 Env (Y710G) was introduced into HIV-1NL4-3 (Fig. 1B) to determine if disruption of this endocytosis motif also increases susceptibility of HIV-1-infected cells to ADCC. Two additional substitutions were tested in HIV-1 gp41, including a tyrosine-to-phenylalanine change (Y710F), previously shown to retain partial endocytic activity (21), and a leucine-to-alanine change in the C-terminal dileucine motif (L852A), also implicated in the endocytosis of Env (Fig. 1B) (22). ADCC assays were performed on HIV-infected target cells using immunoglobulin purified from the

**RESULTS**

A mutation in the membrane-proximal endocytosis motif of SIV gp41 increases the susceptibility of infected cells to ADCC. To assess the influence of potential trafficking motifs on the susceptibility of SIVmac239 to ADCC, CEM.NKR-CCR5-sLTR-Luc cells were infected with either wild-type SIVmac239 or viral mutants carrying a mutation in one of three YXXΦ motifs conserved among the envelope glycoproteins of SIV isolates (Fig. 1A). The tyrosine residue at position 721 of SIV Env was changed to glycine (Y721G), and tyrosine residues at positions 768 and 795 were changed to phenylalanine (Y768F and Y795F) to maintain the amino acid coding sequence of the overlapping rev open reading frame. Infected target cells were incubated with an NK cell line that constitutively expresses rhesus macaque CD16 in the presence of serial dilutions of plasma from an SIV-infected rhesus macaque, and ADCC activity was measured after an 8-h incubation, as previously described (17). Whereas there was no change in susceptibility to ADCC when tyrosine residue 768 or 795 was changed to phenylalanine, a tyrosine-to-glycine change in the membrane-proximal endocytosis motif (Y721G) significantly increased susceptibility to ADCC (Fig. 2A), reducing the antibody titer for half-maximal killing by more than 2 orders of magnitude (Fig. 2F) (P < 0.0001) and resulting in more than a 4-fold difference in area under the ADCC curve (AUC) values (Fig. 2C) (P < 0.0001).

In accordance with the ADCC measurements, Env levels on the surface of virus-infected cells were higher for the Y721G mutant (Fig. 2D). Whereas the geometric mean fluorescence intensity (gMFI) of Env staining was 3.9-fold higher for the Y721G mutant (Fig. 2E) (P = 0.0006), there was no detectable difference in Env staining levels for the Y768F and Y795F mutants (Fig. 2E). Surface expression of Env also correlated with susceptibility to ADCC as measured by the 50% ADCC titer (Fig. 2F) (P < 0.0001) and AUC values (Fig. 2G) (P = 0.0005).
The plasma of HIV-infected donors (HIVIG) and an NK cell line expressing human CD16. The Y710G and Y710F substitutions both resulted in significant increases in susceptibility to ADCC compared to that of cells infected with wild-type HIV-1 (Fig. 3A). HIVIG concentrations for half-maximal ADCC activity were 23-fold and 9-fold lower for Y710G and Y710F, respectively (both, \( P < 0.0001 \)) (Fig. 3B). Likewise, significant differences were detected in AUC values for Y710G (\( P < 0.0001 \)) and Y710F (\( P = 0.0294 \)) (Fig. 3C). The L853A mutant showed an unexpected modest increase in resistance to ADCC, which was significant by comparison of 50% ADCC concentrations (\( P = 0.0406 \)) (Fig. 3B) but not by comparison of AUC values (Fig. 3C).

As observed for mutations in the SIV gp41 tail, Env levels on the surface of cells infected with the HIV-1 gp41 CD mutants correspond to differences in susceptibility to ADCC (Fig. 3D). Surface expression of Env was 2-fold higher for Y710G (\( P = 0.0003 \)) and 1.6-fold higher for Y710F (\( P = 0.0312 \)) than that in cells infected with wild-type HIV-1 NL4-3 (Fig. 3E). However, no difference in Env expression was detected for the L853A mutant (\( P = 0.9175 \)). Env levels were also strongly correlated with susceptibility to ADCC (Pearson correlation test).

FIG 2 The membrane-proximal endocytosis motif of the SIVmac239 gp41 CD protects infected cells from ADCC by downregulating surface Env levels. CEM.NKR-CCR5-sLTR-Luc cells were infected with either wild-type SIVmac239 or a viral mutant containing the indicated substitution in the gp41 tail. Cells infected with SHIV SF162P3 were used to control for nonspecific killing. (A) Infected target cells were incubated with a CD16^+ NK cell line at a 10:1 effector/target ratio in the presence of serial dilutions of plasma from an SIV-infected rhesus macaque. The dose-dependent loss of luciferase activity in percent RLU was used as a measure of ADCC activity, as previously described (17). Error bars represent the standard deviation of triplicate wells, and the dotted line represents 50% ADCC activity. Differences in susceptibilities to ADCC as measured by 50% ADCC titers (B) and AUC values (C) calculated from three independent experiments were compared by one-way ANOVA, corrected for multiple comparisons according to Holm-Sidak. (D) Env levels on the surface of SIV-infected target cells were measured by flow cytometry. The histograms show the fluorescence intensity of Env staining using plasma from an SIV-infected rhesus macaque followed by an anti-human IgG antibody after cells were gated on viable CD45^+ CD4low Gag^+ cells in comparison to that of nonspecific staining in the absence of SIV^+ plasma. Differences in gMFIs of Env staining for three separate experiments were compared by one-way ANOVA with a Holm-Sidak correction for multiple comparisons (E). 50% ADCC titers (F) and AUC values (G) correlate with surface levels of Env (Pearson correlation test).
ceptibility to ADCC, as reflected by the Pearson correlation coefficients for Env staining versus 50% ADCC (Fig. 3F) and AUC values (Fig. 3G).

Disruption of the AP-2-binding site in gp41 and deletion of vpu have an additive effect on susceptibility to ADCC. Previous work by our group and others demonstrated that the loss of Vpu-mediated downregulation of tetherin increases the susceptibility of HIV-1-infected cells to ADCC (12, 13). To investigate whether the protective effect of the conserved AP-2 binding site is cumulative with that of Vpu, we introduced the Y710G substitution into a strain of HIV-1NL4-3 carrying a single nucleotide deletion in vpu.

This mutation results in several stop codons after the fifth codon of vpu and does not alter Env expression levels (12,16). Whereas HIV-1NL4-3 Y710G and HIV-1NL4-3 Δvpu showed similar susceptibilities to ADCC, the combined mutations increased susceptibility to ADCC to a greater extent than either one alone (Fig. 4A). HIVIG concentrations required for 50% killing of HIV-1NL4-3/L9004 vpu Y710G-infected cells were reduced by two orders of magnitude compared to those for wild-type HIV-1-infected cells and 4-fold compared to those for cells infected with either HIV-1NL4-3 Δvpu or HIV-1NL4-3 Y710G (Fig. 4B). Similar differences were observed by comparison of AUC values; cells infected with the combination mutant were 4.5-fold more susceptible to ADCC than cells infected with wild-type HIV-1 and 1.4- and 2.1-fold more susceptible, respectively, than cells infected with the Y710G and Δvpu mutants (Fig. 4C). Changes in Env levels on the surface...
of virus-infected cells as measured by flow cytometry again reflected susceptibility to ADCC (Fig. 4D). Cells infected with HIV-1NL4-3Δvpu Y710G had 3.2-fold higher cell surface Env levels than wild-type-infected cells, a 1.5-fold increase over each separate mutant. Env staining also correlated strongly with both 50% ADCC titers and AUC values (both, *P* < 0.0001, Pearson correlation test) (Fig. 4F and G).

The membrane-proximal endocytosis motif protects primary HIV-1 isolate-infected cells from ADCC. To determine the impact of Env endocytosis on the susceptibility of cells infected with a primary HIV-1 isolate to ADCC, we introduced a corresponding tyrosine-to-glycine substitution at position 704 in gp41 of HIV-1JR-CSF (Fig. 1B). ADCC activity was tested using eCD4-IgG (Fig. 5A), a CD4-Ig fusion with the CCR5-mimetic sulfopeptide CCR5mim2 that has potent broadly neutralizing activity against HIV-1 (19, 20). Although 50% lysis was not achieved in all experiments, AUC values for ADCC activity against HIV-1JR-CSFY704G-infected cells were 10-fold higher than for activity against cells infected with wild-type HIV-1JR-CSF (*P* = 0.0089) (Fig. 5B). These findings were mirrored by cell surface Env levels (Fig. 5C), which were increased 3.9-fold by disrupting the membrane-proximal AP-2 binding site of HIV-1JR-CSF gp41 (*P* = 0.0001) (Fig. 5D). A significant
correlation was also detected between Env levels and AUC values (Fig. 5E) by the Pearson correlation test \( (P = 0.0029) \).

**DISCUSSION**

Here, we show that disruption of the conserved membrane-proximal AP-2 binding site in the gp41 cytoplasmic tail increases the sensitivity of HIV-1- and SIV-infected cells to antibody-dependent cell-mediated cytotoxicity. Greater susceptibility to ADCC correlated with increased surface expression of Env and was additive with a deletion in vpu previously shown to enhance the susceptibility of HIV-1-infected cells to ADCC (12, 13, 15). These results support a role for the membrane-proximal endocytosis motif of gp41 in protecting HIV-1- and SIV-infected cells from ADCC by minimizing the exposure of the viral envelope glycoprotein on the cell surface prior to the assembly and release of virus particles. It is important to note that while this study assessed susceptibility to ADCC, the regulation of Env expression on the surface of virus-infected cells may also provide resistance to other Fc receptor (FcR)-mediated functions of antibodies. In addition to ADCC, Env-specific antibodies may contribute to the elimination of HIV-1- and SIV-infected cells *in vivo* by complement fixation and by FcR-dependent phagocytosis. Thus, Env internalization is likely to have a broader role in lentiviral resistance to antibody responses directed against virus-infected cells than protection against ADCC alone.

Recent studies have also identified other mechanisms by which HIV-1-infected cells evade antibody responses. We, along with others, demonstrated that Vpu protects HIV-1-infected cells from ADCC by counteracting restriction by tetherin (12, 13), an interferon-inducible transmembrane protein that inhibits virus release from infected cells (23). By preventing tetherin-mediated accumulation of budding virions on the cell surface, Vpu reduces the binding of Env-specific antibodies capable of directing the killing of infected cells by ADCC. Nef- and Vpu-mediated downmodulation of CD4 also affords resistance to ADCC (14, 15). In this case, the loss of these viral gene products enhances the susceptibility of HIV-1-infected cells to antibodies specific for epitopes of gp120 normally occluded in the native Env trimer (14). Thus, CD4 downmodulation by Nef and Vpu appears to prevent the elimination of infected cells by antibodies directed to gp120 surfaces exposed by the formation of gp120-CD4 complexes at the plasma membrane. Together with the results reported here, these observations help to explain why antibody responses directed against virus-infected cells, like other mechanisms of immunity, ultimately fail to contain HIV-1 replication in chronically infected individuals.

We also tested mutations in potential trafficking motifs besides
the membrane-proximal AP-2 binding site (24, 25). While there is evidence that the conserved C-terminal dileucine motif has some effect on endocytosis of Env in HIV-1-infected cells (22), it does not impair virus infectivity (26) and has little effect on Env internalization by itself (27). Thus, it is perhaps not surprising that we saw no effect on surface Env levels and only small changes in susceptibility to ADCC when it was disrupted. Likewise, although previous findings describe no effect on endocytosis mediated by the additional YXXΦ motifs at positions 768 and 795 in SIVmac239 (2, 28), we decided to include them in our study because they are highly conserved among SIV isolates (25). In accordance with existing literature, we observed no significant effect on cell surface Env levels or susceptibility to ADCC when these motifs were disrupted. Our results therefore suggest that these motifs do not play a major role in the resistance of virus-infected cells to ADCC.

This study describes a fundamental new role for the highly conserved endocytosis motif in the cytoplasmic tail of gp41 in protecting HIV-1- and SIV-infected cells from elimination by antibodies. Together with recent evidence that Vpu-mediated down-regulation of tetherin and Nef-mediated downregulation of CD4 afford resistance to ADCC (12–15), the picture that emerges is that the primate lentiviruses have acquired multiple complementary mechanisms to reduce the susceptibility of virus-infected cells to antibodies. These findings have important practical implications since they suggest that approaches for preventing Env internalization may enhance the efficacy of antibody-based vaccines and therapies designed to improve the immunological containment of HIV-1 replication in chronically infected individuals.

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