A region of the C-terminal tail of the gp41 envelope glycoprotein of human immunodeficiency virus type 1 contains a neutralizing epitope: evidence for its exposure on the surface of the virion

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INTRODUCTION

The ~150 amino acid C-terminal tail of the gp41 transmembrane glycoprotein of human immunodeficiency virus type 1 (HIV-1) is generally thought to be located inside the virion. However, we show here that both monoclonal IgG and polyclonal epitope-purified IgG specific for the 746ERDRD750 epitope that lies within the C-terminal tail neutralized infectious virus. IgG was mapped to the C-terminal tail by its failure to neutralize tail-deleted virus, and by sequencing of antibody-escape mutants. The fact that antibody does not cross lipid membranes, and infectious virus is by definition intact, suggested that ERDRD was exposed on the surface of the virion. This was confirmed by reacting virus and IgG, separating virus and unbound IgG by centrifugation, and showing that virus was neutralized to essentially the same extent as virus that had been in constant contact with antibody. Epitope exposure on virions was independent of temperature and therefore constitutive. Monoclonal antibodies specific to epitopes PDRPEG and IEEE, upstream of ERDRD, also bound to virions, suggesting that they too were located externally. Protease digestion destroyed the ERDRD and PDRPEG epitopes, consistent with their proposed external location. Altogether these data are consistent with part of the C-terminal tail of gp41 being exposed on the outside of the virion. Possible models of the structure of the gp41 tail, taking these observations into account, are discussed.

The gp160 envelope polypeptide of human immunodeficiency virus type 1 (HIV-1) is a type I glycoprotein which, during formation of the virion, is cleaved into a surface (SU) domain (gp120) and a transmembrane (TM) domain (gp41) that is essential for fusion activity and virion infectivity. The mature gp41 is a homotrimer (Blacklow et al., 1995; Lu et al., 1995), and divided by a transmembrane region into an extracellular (N-terminal) ectodomain and a C-terminal region. At the N terminus is a hydrophobic peptide (amino acids 519–534; numbering system of Ratner et al., 1985) that inserts into the plasma membrane of the target cell after attachment of virus, and results in fusion of the viral and target cell membranes and virus entry. A crystal structure has been determined for the ectodomain only (Chan et al., 1997; Weissenhorn et al., 1997). There are three other hydrophobic regions in gp41: the transmembrane region (691–712), and two amphipathic regions [lentiviral lytic peptide (LLP)-2: 777–797 and LLP-1: 835–855]. The latter associate with cell membranes (Eisenberg & Wesson, 1990; Haffar et al., 1988, 1991), and may be transmembrane or associated with the inner leaflet of the lipid bilayer (Venable et al., 1989). Synthetic peptides derived from these regions compromise membrane integrity and function by permeabilizing membranes, and forming pores and possibly ion channels (Arroyo et al., 1995; Chernomordik et al., 1994; Comardelle et al., 1997; Gawrisch et al., 1993; Miller et al., 1991, 1993; Srinivas et al., 1992; Zhang et al., 1996).

There are several antigenic sites within gp120 and gp41 that correlate with virus neutralizing ability (reviewed by Burton, 1997). 669ELDKWA677 is an important cross-clade reactive epitope in the ectodomain of gp41 (Muster et al., 1993; Sattentau et al., 1995), while 746ERDRD750 is situated in a supposedly intravirion location within the 731PRGPDRPEGIEEEGERDRDRS752 peptide (Gallagher
et al, 1992; Kennedy et al, 1986; Modrow et al, 1987), although its location inside the virion is controversial (Buratti et al, 1997; Dalgleish et al, 1988; Kennedy et al, 1986; Niedrig et al, 1992; Sattentau et al, 1995). If it was accepted that the 731–752 peptide contains a neutralizing epitope, this would almost certainly mean that the part of the C-terminal tail containing that epitope was looped back through the viral membrane to the exterior in free virions, or that it was normally hidden and was exposed intermittently. However, only weakly or non-neutralizing monoclonal antibodies (mAbs) have been raised (Dalgleish et al, 1988; Niedrig et al, 1992; Vella et al, 1993), so the function of region 731–752 as a neutralization site is regarded with some scepticism. Nonetheless, we show here that one of the ERDRD-specific mAbs neutralized virus in the presence of complement.

The antigenic properties of gp41 region 731–752 were first described by Kennedy et al (1986). The antibody response to this region in infected patients is generally poor (Davis et al, 1990; Niedrig et al, 1992; Vella et al, 1991), although several groups have prepared 731–752 peptide-specific neutralizing antisera using antigen-presenting systems (Evans et al, 1989; McLain et al, 1995, 1996a; Newton et al, 1995) or synthetic peptides (Chanh et al, 1986). However, this is not always the case (Kalyan et al, 1994). Indeed, these and other immunological assays suggested that mAbs to the Kennedy peptide might react with HIV-1 particles, although interaction with broken virus or virus after it had attached to cell receptors could not be excluded (Niedrig et al, 1992). Some of these apparently conflicting data were resolved with the realization that the Kennedy peptide contained, not one, but three epitopes: 734PDRPEG753 (Abacioglu et al, 1994), 742IEEE743 and 746ERDRD750 (Vella et al, 1993).

We have recently confirmed the presence of the IEEE and ERDRD epitopes in gp41 using epitope-purified polyclonal antibodies, but only antibody to ERDRD, a conformationally constrained epitope, was neutralizing. IEEE is a linear epitope and its cognate antibodies are non-neutralizing (Buratti et al, 1998). mAb C8 usually recognizes a denatured form of the PDRPEG epitope (Abacioglu et al, 1994), but also reacts with virions that have escaped neutralization with ERDRD-specific antibody (McLain et al, 2001). It is non-neutralizing. The IEEE sequence is both immunogenically and antigenically dominant over the ERDRD epitope, and this has provided an explanation for some of the confusion surrounding the immunology of the Kennedy peptide (Cleveland et al, 2000a).

In this report we present data from a variety of approaches suggesting that a region of gp41, C-terminal to the main transmembrane region, is exposed on the exterior of infectious virions, possibly as a loop supported by two additional transmembrane regions. We therefore suggest that the structure of the HIV-1 gp41 protein should be revisited. Implicit in our findings are previously unsuspected HIV-1 functions mediated through the gp41 tail loop, and a new target for immunological and chemotherapeutic antiviral measures.

METHODS

Cells and viruses. The human T cell lines H9, MT4 and C8166 (AIDS Reagent Project, NIBSC, Potters Bar, UK) were grown in RPMI 1640 medium without antibiotics (Life Technologies), supplemented with 2 mM glutamine and 10 % (v/v) heat-inactivated foetal calf serum (LabTech International). HIV-1 IIIB was produced in H9 cells. Medium was replaced 24 h before harvesting, and virus stored in liquid nitrogen. HIV-1 HXB10 and its deletion mutant (ΔCT) that lacks the C-terminal 144 amino acids of gp41 (Mammano et al, 1995) were kindly donated by H. Göttlinger (Boston, Massachusetts, USA). Stocks were prepared in MT4 cells. When required virus was purified on a 15–60 % sodium/potassium tartrate density gradient.

Antibodies. Neutralizing antigen was purified by immunizing 6–8-week-old C3H/He-mg mice (H-2k; bred in-house) with CPMV-HIV/1 expressing the HIV-1 gp41 peptide 731PRGPDPERGEEEEGERDRD752 (McLain et al, 1995). Mice were injected subcutaneously on days 0 and 28 with 10 µg CPMV-HIV/1 or CPMV in aluminium hydroxide (Imject Alum: Pierce & Warriner), and bled on day 42. Other gp41 antibodies used were: mouse mAb 1575 to 742IEEE743; mAbs 1577 1578 and 1583 to 746ERDRD750 (Vella et al, 1993), mAb C8 to 734PDRPEG753 (Abacioglu et al, 1994), and the human mAb 2F5 to amino acids 669ELDKWA764 (Muster et al, 1993). gp120 antibodies were the human CD4-binding site mAb b12 (Burton et al, 1994), and the V3 rat mAb ICR41.1 (McKeating et al, 1992).

Epitope-purified ERDRD-specific (EPES) IgG was prepared by adsorption to and elution from FHV-L1-B, a flock house virus fusion protein that presents the gp41 sequence GERDDR in its neutralizing conformation (Buratti et al, 1998). Wild-type FHV protein did not adsorb neutralizing antibody. Purified FHV-L1-B was produced in Escherichia coli as previously described, and immobilized on nitrocellulose (Buratti et al, 1996). ERDRD-specific IgG was adsorbed and then eluted with glycerine buffer solution, pH 2-5. This was predominantly IgG1, IgG2a and IgG2b (Stratagene IsoDetect). IgG was quantified using a solid-phase goat anti-mouse IgG ELISA or by determining the OD280.

Assay of HIV-1 neutralizing antibody. Neutralization of all HIV-1 strains was measured by inhibition of infectious progeny production, p24 antigen production or syncytium formation using C8166 cells, as indicated in the text (Buratti et al, 1998; McLain & Dimmock, 1994). Antibody was incubated with 2000 syncytium-forming units (s.f.u.) ml-1 HIV-1 for 1 h at 37°C, and for complement-mediated neutralization was further incubated with an optimized dilution of guinea pig complement (Gibco BRL) for 1 h at 37°C. C8166 cells (2 × 104) were infected for 1 h at 37°C, washed and incubated for 3 days at 37°C. Production of infectious progeny at 3 days after infection was determined by infectivity titration. P24 was captured from Empigen (Calbiochem)-treated TCF with solid-phase sheep anti-p24 antiserum (Aalto Bioreagents), and detected with a biotinylated mouse anti-p24 mAb (AIDS Reagent Project) by standard methodology. Syncytium production was determined by counting 50–100 syncytia. Neutralization was calculated as the percentage reduction of infectivity due to antibody compared to that of non-neutralized virus.

Western blot of HIV-1 HXB10 and HIV-1 ΔCT-infected MT4 cells. Virus or infected cells were boiled in reducing buffer [10 mM Tris pH 7-4, 5 % (w/v) dithiothreitol, 0-2 % (w/v) SDS, 7-5 % (w/v) glycerol and 0-004 % (w/v) bromophenol blue] for 2 min, and electrophoresed on a 10–30 % SDS-PAGE gel. Polypeptides were transblotted to nitrocellulose, blocked and incubated with antibody in 10 % (w/v) defatted dried milk in TBS (20 mM Tris, 140 mM NaCl, pH 7-6). Bound antibody was detected with anti-species IgG–horse radish peroxidase (HRP; Bio-Rad) and 3,3′-diaminobenzidine (Sigma).
Binding of ERDRD-specific IgG to virus in solution. Purified HIV-1 (5 × 10⁶ s.f.u.) was incubated with 2 µg EPES IgG for 1 h at 37°C. The virus–antibody mix was then layered onto 4.5 ml of 20% sucrose in TBS, and centrifuged at 155,000 g for 1 h to separate virus or putative virus–antibody complexes from free antibody, which remains at the top of the tube (Jackson et al., 1999). The supernatant was carefully removed from the top, avoiding contamination of the virus pellet with free antibody. The pellet was resuspended and assayed for infectivity or the presence of bound antibody. For the latter, virus was heat inactivated in 1% Empigen at 56°C for 1 h, and incubated with solid-phase anti-species IgG in TBS containing 0.5% BSA. Bound primary antibody was detected with biotinylated anti-species IgG, streptavidin-conjugated alkaline phosphatase, and a p-nitrophenyl phosphate substrate. There was no significant cross-reaction between human and mouse IgGs.

Binding of antibodies to paraformaldehyde-fixed virus. Purified HIV-1 was fixed with 2% paraformaldehyde and 3 × 10⁴ s.f.u. captured using solid phase-bound human mAb b12 (0.1 µg per well). After washing, virus was incubated with a non-human gp120- or gp41-specific antibody, using normal IgG as a control. Binding of IgG was detected using species-specific anti-IgG as described above.

Removal of virus surface proteins with proteases. Purified virus was incubated with 20 mg trypsin (Sigma) ml⁻¹ for 30 min at 37°C. The reaction was stopped by the addition of 20% (v/v) foetal calf serum. Alternatively, virus was incubated with 10 µg thermolysin (Calbiochem) ml⁻¹ for 1 h on ice, before terminating the reaction with 50 mM EDTA. Digested virus particles and free protein were separated by centrifugation. P24 antigen assay showed there was no loss due to protease digestion. Virus for electron microscopy was stained with 4% sodium silicotungstate, pH 6-9.

RESULTS

Neutralization of HIV-1 by monoclonal and affinity-purified polyclonal ERDRD-specific IgGs

Currently, there are no mAbs specific for the ERDRD epitope that are capable of independent neutralization (Vella et al., 1993). However, relatively high antibody concentrations (60 µg ml⁻¹) of mAb 1577 were capable of significant (60%) neutralization in the presence of complement (Fig. 1a). Since this was not an amenable system, we continued with ERDRD-specific antiserum (Fig. 1b) and epitope-purified ERDRD-specific (EPES) IgG (Fig. 1c). We used the latter in three different neutralization assays (inhibition of p24 antigen production, inhibition of the production of infectious progeny or inhibition of cytopathogenicity of this syncytium-forming virus). All assays gave approximately 90% neutralization at around 3 µg ml⁻¹ and 50% neutralization at about 0.3 µg ml⁻¹ (Fig. 1c).

Mapping of ERDRD-specific IgG by failure to neutralize a gp41 tail-deleted virus (∆CT)

Although the ERDRD-specific IgG was affinity-purified from the FHV-L1-B fusion protein that expresses GERDRDR as its only HIV-1-specific sequence, its neutralizing epitope had not been definitively located on HIV-1. While there is no other linear ERDRD motif in the HIV-1 envelope protein, it was possible that EPES IgG binds to a discontinuous epitope mimic on either gp120 or gp41. This
problem was addressed using a mutant of HXB10 that lacks the C-terminal 144 residues of gp41 and has a tail of just seven residues (ΔCT: Mammano et al., 1995). Initially, we confirmed that wt and ΔCT virus were both neutralized equally well by gp120-specific antibodies (L. Cheung & N. J. Dimmock, unpublished data). We then determined the authenticity of ΔCT virus. The latter is evidenced by the faster migration of the truncated gp160, and its failure to Western blot with IgG specific for epitope740IEEE743, located in the gp41 tail (Fig. 2a). The gp41 incorporated into ΔCT virions also was reduced in size (Fig. 2b). Finally, Fig. 2(c) shows that the EPES IgG solidly neutralized HXB10 but gave no neutralization of ΔCT virus, thus locating its epitope to the C-terminal tail of gp41.

IgG complexes with and neutralizes infectious HIV-1 virions

We have demonstrated so far that EPES IgG recognizes the C-terminal tail of gp41 that is viewed conventionally as being inside the virion, and mediated neutralization through it. In addition, EPES antibody selected neutralization escape mutants with a residue substitution in the same region of the tail that abrogated binding by the selecting antibody (McLain et al., 2001). Thus the ERDRD epitope appears to be exposed on the surface of the virion at some stage of the virus life-cycle. The most likely scenarios are that the gp41 tail is (a) normally internal but extruded when virus reacts with cell receptors, or (b) constitutively external. Either scenario means that there would have to be an additional transmembrane domain conducting the C-terminal tail to the exterior. Since virus preparations can never be guaranteed to be free of broken particles in which the gp41 tail could be artefactually exposed, we used infectivity, the gold standard for virion intactness, to determine when the gp41 tail was exposed.

In the experiment shown in Fig. 3, purified virus alone, or virus mixed with various amounts of EPES IgG, was incubated for 1 h at 37˚C. Putative virus–antibody complexes and virus were then separated from free antibody by layering over sucrose and centrifuging. The virus pellet was then resuspended and the extent of neutralization determined. Any unattached virus/virus–antibody complexes or free antibody were removed by washing cells after a 1 h adsorption period at 37˚C. We found that all antibody concentrations tested were neutralizing, and that the extent of neutralization was only slightly less than virus neutralized by the standard procedure (virus + antibody with no centrifugation) (Fig. 3). For example, at the highest antibody concentration there was 82 % neutralization of centrifuged virus–antibody complexes and 88 % neutralization by the standard procedure. This demonstrated that the ERDRD epitope was exposed on the outer surface of free virus particles and was capable of binding its cognate neutralizing antibody.

Controls ensured that there was no significant contact between the pelleted virus and free antibody that remained at the top of the gradient. We showed that antibody alone did not pellet, and that no antibody contaminated the virus pellet during the sampling procedure. To demonstrate the

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**Fig. 2.** Mapping of the ERDRD epitope to the C-terminal tail of gp41. (a) PAGE and Western blotting of MT4 cells infected with HIV-1 HXB10 or HXB10ΔCT. HXB10 has a full-length gp160, and ΔCT lacks all but seven residues of the C-terminal tail of gp41, and its truncated envelope protein migrates faster. The uncleaved envelope polypeptides (arrows) of both viruses react with mAb 2F5 that recognizes an epitope in the ectodomain of gp41. The HXB10 gp160 but not that of HXB10ΔCT was recognized by antiserum to the linear 740IEEE743 epitope of gp160 that is adjacent to 746ERDRD750. Preimmune serum was negative. (b) PAGE of purified virus and Western blot with mAb 2F5, showing that the HXB10ΔCT gp41 (solid arrow) migrated faster than that of wt HXB10 virions (open arrow) as expected. (c) EPES IgG neutralized only HIV-1 that has a complete gp41 C-terminal tail. ■, Neutralization of HXB10; ●, lack of neutralization of HXB10ΔCT virus. Virus and antibody were incubated for 1 h at 37˚C before assaying the inhibition of syncytium formation using C8166 cells. Experiments were carried out at least twice and the bar is the SEM.
then layered antibody over the gradient, and removed the supernatant. There was no neutralization of virus recovered from the pellet (data not shown).

The gp41 ERDRD epitope is exposed on virions at 4, 21 and 37°C

The previous experiment was repeated using an ELISA format to determine if the exposure of the ERDRD epitope was temperature-dependent. Virus and EPES IgG were incubated together, and putative virus–antibody complexes separated from free antibody by centrifugation through a sucrose spacer as described above. Similar amounts of ERDRD antibody were bound at 4°C (120%), 21°C (133%) and 37°C (100%). Thus the ERDRD region is exposed over a wide temperature range.

The gp41 ERDRD epitope on virus particles is sensitive to protease digestion

If the ERDRD epitope is exposed on the surface of virus as the data above suggest, it should be sensitive to digestion by proteases. Virtually complete removal of surface proteins from purified virus, as judged by PAGE analysis, was achieved by incubation with trypsin or thermolysin. Electron microscopy (Fig. 4a) showed that trypsin-digested

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Fig. 4. The ERDRD and PDRPEG epitopes in the Kennedy sequence of gp41 are destroyed by incubation of purified virus with proteases. (a) Electron micrographs of representative virus particles before (upper) and after (lower) trypsin treatment. Virus alone or virus + 20 mg trypsin ml⁻¹ was incubated for 30 min at 37°C, purified by centrifugation on a tartrate gradient, and stained with 4 % sodium silicotungstate, pH 6.9. The bar represents 50 nm. (b) Binding of antibodies to control (non-trypsin-treated) virus (−) or trypsin-treated virus ( + ). Non-treated and trypsin-treated virus were standardized by ELISA for their internal virion p24 antigen, and incubated in solution with EPES IgG or mAb 2F5 to the main ectodomain of gp41. Virus or virus–antibody complexes were then separated from unbound antibody by centrifugation through a 20 % sucrose spacer (see Fig. 3) and bound IgG was assayed by ELISA. (c) Western blot of thermolysin-treated virus (T), mock-treated virus (M) and untreated virus (V). Purified virus was digested with thermolysin (10 μg ml⁻¹) for 1 h on ice. Virus preparations (T, M) were centrifuged free of soluble protein. No p24 was lost from the thermolysin-digested virus. Virion polypeptides were then separated by PAGE on a 15 % reducing gel. After transblotting, gp41 was identified by reaction with mAb C8 to epitope PRDREG and mAb 2F5 to the ectodomain of gp41. Data are representative of at least two experiments.

latter we pelleted virus in the absence of antibody and then layered antibody over the gradient, and removed the supernatant. There was no neutralization of virus recovered from the pellet (data not shown).
particles were smooth and disaggregated whereas control particles had visible spikes/protrusions, and also tended to clump together. Virus digested in this way was then re-banded, and amounts of protease-treated virus and non-protease-treated control virus standardized by ELISA according to their internal virion p24 antigen content. These were then incubated with EPES IgG and virus/virus–antibody complexes separated from free antibody by centrifugation through a sucrose spacer. Antibody bound to virions was detected by ELISA using solid-phase anti-mouse IgG, MAb 2F5, which recognizes an epitope in the ectodomain of gp41, was used in parallel. Digestion of HIV-1 with trypsin reduced the binding of EPES IgG to virus by 93.5 % and of MAb 2F5 by a similar amount (81 %) (Fig. 4b). In an experiment with another protease, digestion with thermolysin destroyed all reactivity of MAb C8 to 734PDRPEG739, an upstream epitope, in Western blots (Fig. 4c). The internal virion p24 protein was unaffected by protease. Data are consistent with the exposure of the PDRPEG and ERDRD epitopes on the surface of intact virions.

Reaction with antibodies defines a number of gp41 C-terminal tail epitopes that are exposed on the outside of the virion

Antibodies to sites N-terminal and C-terminal to the neutralizing ERDRD epitope were reacted with native or fixed virions to determine the extent to which the C-terminal tail of gp41 was exposed on the outer surface of the virion. Assays were done as follows.

**Binding of monoclonal and polyclonal ERDRD-specific antibodies to infectious virus.** Antibody was reacted with purified virus, and putative virus–antibody complexes separated from free antibody by centrifugation through sucrose. Virus was then disrupted with detergent and bound IgG captured and assayed. Fig. 5(a) shows that virions bound 7-fold more mAb 1583 (ERDRD-specific) than normal mouse IgG, and 12-fold more EPES IgG. Binding of MAb 2F5 to the ELDKWA epitope in the main ectodomain of gp41 (Muster et al., 1993) is also shown. The integrity of virions was evidenced by their failure to bind p17-specific and p24-specific antibodies (data not shown).

**Binding of ERDRD- and IEEE-specific mAbs and of epitope-purified ERDRD-specific IgG to paraformaldehyde-fixed virus.** Purified virus was fixed with paraformaldehyde at 4 °C as an additional precaution against disruption of virus integrity. Virus was then bound to plastic with gp120-specific human IgG (mAb b12). Binding of the ERDRD-specific mAb 1583 was unaffected, and we also demonstrated binding of the IEEE-specific mAb 1575, and

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**Fig. 5.** Gp41 Kennedy sequence-specific monoclonal and polyclonal IgGs bind to native or paraformaldehyde-fixed HIV-1 virions. Purified virus was pelleted and resuspended in PBS (a) or fixed in 2 % paraformaldehyde overnight at 4 °C (b). All reactions with IgG were for 1 h at 37 °C. In (a) putative virus–IgG complexes were separated from unbound antibody by centrifugation through 20 % sucrose as described in Fig. 3 and lysed. IgG that had been bound to virus was captured using immobilized goat anti-mouse IgG and quantified by ELISA. In (b) fixed virus was captured using immobilized gp120-specific human mAb b12 (4 °C overnight), before being reacted with envelope protein-specific rodent IgGs. Bound IgGs were again quantified using anti-species IgG and ELISA. In (a) we used the ERDRD-specific mAb 1583, polyclonal EPES IgG, pre-immune mouse IgG and ELISA. In (b) we used the ERDRD-specific mAb 1583, polyclonal EPES IgG, human MAb 2F5 (to epitope ELDKWA in the main ectodomain of gp41) and normal human IgG (all 3 µg ml–1). In (b) we titrated MAb 1583 at the concentrations (µg ml–1) shown. Values for the binding of equivalent amounts of normal mouse IgG (e.g. 0.06 OD for 0.6 µg ml–1) have been subtracted. EPES IgG was used at 0.25 µg ml–1, the IEEE-specific mAb 1575 at 10 µg ml–1, and the gp120 V3-specific mAb 41.1i at 3 µg ml–1. In all experiments the SD was <0.1 OD.
EPES neutralizing polyclonal IgG (Fig. 5b). The binding of the gp120 V3 loop-specific mAb ICR41.1i is also shown.

**DISCUSSION**

MAb to ERDRD with complement, or EPES IgG, neutralized HIV-1 through an epitope situated in a C-terminal region of gp41 that was previously thought to be inside the virion (Fig. 1). The epitope was mapped by failure of the antibody to neutralize a gp41 C-terminal tail deletion mutant (Fig. 2), through neutralization/binding antibody-escape mutants that have an upstream $^{73}_{73}R\rightarrow G$ substitution (McLain et al., 2001), and the blocking of neutralization via ERDRD by a mAb to the adjacent IEEE epitope (Cleveland et al., 2000a). Antibody bound to and neutralized HIV-1, demonstrating unequivocally that epitope ERDRD was normally exposed on the outside of infectious, intact virions (Fig. 3). Epitope exposure was independent of temperature, unlike some poliovirus epitopes that are exposed only at physiological temperature (Li et al., 1994). Abrogation of the binding of $^{73}_{73}PDRPEG^{739}$ or $^{74}_{74}ERDRD^{750}$-specific IgG by prior digestion of virus with proteases was consistent with exposure of the epitopes on the outside of the virion (Fig. 4). However, failure of antibodies made to peptides 799–817 and 844–863, C-terminal to ERDRD, bind to virions (data not shown), suggested that these regions were unavailable to antibody and possibly inside the virion, thus making ERDRD part of a loop structure.

**Immunological implications**

One of the stumbling blocks to the appreciation that the Kennedy sequence of gp41 is part of an external loop structure was conflicting evidence about its ability to stimulate neutralizing antibodies. While we have consistently found that the gp41 731–752 peptide, expressed on the surface of chimeric CPMV-HIV/1 particle elicited neutralizing antibodies in mice (Durrani et al., 1998; McLain et al., 1999; McLain et al., 1995, 1996a, b), other formats have been less successful (Chanh et al., 1989; Evans et al., 1989), and only weakly or non-neutralizing mAbs have been raised (Dalgleish et al., 1988; Evans et al., 1989; Niedrig et al., 1992; Pincus et al., 1993; Vella et al., 1993). Others have been unable to raise neutralizing polyclonal antibodies (Newton et al., 1995; Pincus et al., 1993), possibly because the ERDRD epitope was presented in a non-neutralizing conformation. However, the situation is more complex. In the first place, the Kennedy sequence in wild-type virions contains two functional epitopes, the linear IEEE epitope that elicits non-neutralizing antibodies, and the conformational ERDRD epitope that elicits neutralizing epitopes (Buratti et al., 1998; Vella et al., 1993). A third epitope, PDRPEG, is normally only detected by mAb C8 in Western blots (Abacioglu et al., 1994), although it reacts with neutralization escape mutants selected with ERDRD-specific antibody (McLain et al., 2001). Secondly, the IEEE epitope and upstream sequence expressed in the CPMV-HIV/1 chimera suppresses the production of neutralizing ERDRD-specific IgG (Cleveland et al., 2000a). In rabbits, the immunogenic dominance of the IEEE epitope was so strong that no neutralizing or ERDRD-specific antibodies were detected at any time in animals immunized repeatedly with adjuvanted CPMV-HIV/1. However, rabbits immunized with another chimera, CPMV-HIV/29, that expresses a truncated form of the 731–752 peptide, GERDRDR, readily made ERDRD-specific neutralizing IgG. The situation was similar, but less extreme, in mice. Thirdly, IEEE-specific antibodies were antigenically dominant over ERDRD-specific antibodies, preventing them binding to their epitope, and blocking neutralization of HIV-1 (Cleveland et al., 2000a). Thus much of the conflicting data in the literature can be reconciled by our recent findings.

**Structural implications**

Data in this report suggest that the currently held view that the C-terminal tail of gp41 is contained entirely within the HIV-1 virion (e.g. Levy, 1998) is mistaken, and that a loop of gp41, C-terminal to the current transmembrane region, is exposed on the outside of the virion. Alternatively, the tails of only some virion envelope proteins may be exposed, while others are entirely intravirion. To have an exposed C-terminal loop, gp41 must cross the virion lipid bilayer an even number of times before the start of the exposed section. In addition, data showing that the gp41 tail interacts with the p17 MA protein suggest that a portion of tail is inside the virion (Bukrinskaya & Sharova, 1990; Cosson, 1996; Dorfman et al., 1994; Freed & Martin, 1995a, b, 1996; Mammano et al., 1995; Murakami & Freed, 2000a; Wyma et al., 2000). Supporting evidence comes from the failure of antibodies to regions 799–817 and 844–863 to react with virions, suggesting that these sequences are inside the virion (unpublished data). If this is the case, gp41 must cross the lipid bilayer of the virion at least once more, making a minimum of three transmembrane regions, as we suggested earlier (McLain et al., 2001). This proposal is consistent with secondary structural predictions. Using standard algorithms and a moving window of 7 amino acid residues, we found, as others have done before us, that the current transmembrane region (691–712) has a significant bimodal hydropathicity (Eisenberg et al., 1995; Murakami & Freed, 2000a; Freed & Martin, 1995a, b). Other transmembrane regions, however, do not show the characteristic features of transmembrane regions. While there is a peak of hydropathicity at 755–763 and 766–772 (Venable et al., 1989), the first transmembrane region (691–712) has a significant bimodal hydropathicity (Eisenberg et al., 1995; Murakami & Freed, 2000a; Freed & Martin, 1995a, b). Other transmembrane regions, however, do not show the characteristic features of transmembrane regions. While there is a peak of hydropathicity at 755–763 and 766–772 (Venable et al., 1989), the first transmembrane region (691–712) has a significant bimodal hydropathicity (Eisenberg et al., 1995; Murakami & Freed, 2000a; Freed & Martin, 1995a, b). Other transmembrane regions, however, do not show the characteristic features of transmembrane regions. While there is a peak of hydropathicity at 755–763 and 766–772 (Venable et al., 1989), the first transmembrane region (691–712) has a significant bimodal hydropathicity (Eisenberg et al., 1995; Murakami & Freed, 2000a; Freed & Martin, 1995a, b).
minimum of seven residues to cross the hydrophobic core of the membrane (Schirmer, 1998; Schirmer & Cowan, 1993).

Fig. 6 summarizes data showing that epitopes $^{734}$PDRPEG$^{739}$, $^{740}$IEEE$^{743}$ and $^{746}$ERDRD$^{750}$ are exposed on the outside of virions. Since these epitopes appear to be situated in an external hydrophilic loop structure, there must be three (Fig. 7a), or possibly four (Fig. 7b), transmembrane domains to take the gp41 C-terminal tail out of the virion and then back again. The exact size of the loop is not known, as it depends on the number of residues that are exposed between the proposed tm 2 region and the $^{734}$PDRPEG$^{739}$ epitope, and the position of tm 3 on the C-terminal side of the loop. The transmembrane domains in Fig. 7(a) could be either short $\alpha$-helices or $\beta$-strands as discussed above. We have not specified the tm 3 sequence in Fig. 7(a) as there are at least two candidates (see above). However it is possible, as shown in Fig. 7(b), that tm 3 and tm 4 are derived from LLP-2 and LLP-1 respectively, and are $\alpha$-helices of about 20 residues. These have been suggested to interact in an anti-parallel charge-complementary fashion (Venable et al., 1989). Tm 4 would take the extreme C-terminal region back into the membrane and form an intravirion, closed loop.

**Fig. 6.** Sequence of the gp41 C-terminal tail region showing the main epitopes and other features of interest. The currently accepted single transmembrane region (note the positively charged arginine residue in its centre) is indicated in italics. Epitopes are in colour. Data for the binding of antibodies to virions are from Figs 1–5, and positive antibody reactions are designated ‘+’. This suggests that the epitopes are exposed on the outside of the HIV-1 virion. The hydrophobic LLP-2 and LLP-1 sequences are also italicized.

**Fig. 7.** Putative models consistent with data derived in this report suggesting that part of the C-terminal tail of gp41 is exposed on the exterior of the virion. These are shown with (a) three or (b) four transmembrane domains (tm 1–4) supporting an external loop of about 40 residues. The tm 3 sequence is left unspecified in (a) or suggested to be in LLP-2 in (b) (see text). The locations of the neutralizing ERDRD epitope, the upstream non-neutralizing PDRPEG and IEEE epitopes, and the downstream and the hydrophobic LLP-2 and LLP-1 sequences are also indicated.

**Functional implications**

Clearly, our suggestion that part of the gp41 tail is looped out on the surface of the virus has wide-ranging implications for its many properties. For example, the C-terminal domain of gp41 of HIV-1, HIV-2 or simian immunodeficiency virus is involved in incorporation of the envelope
protein into virions (Celma et al., 2001; Iwatani et al., 2001; Manrique et al., 2001; Murakami & Freed, 2000b; Piller et al., 2000; Yu et al., 1993; Zingler & Littman, 1993), fusogenicity (Mulligan et al., 1992; Sodroski et al., 1986; Wilk et al., 1992; Zingler & Littman, 1993), infectivity (Celma et al., 2001; Iwatani et al., 2001; Piller et al., 2000) and interaction with the p17 MA protein (Bukrinskaya & Sharova, 1990; Cosson, 1996; Dorfman et al., 1994; Freed & Martin, 1995a, b, 1996; Mammano et al., 1995; Murakami & Freed, 2000a; Wyma et al., 2000). Our model of the structure of the C-terminal tail of gp41 impacts on the internalization of plasma membrane inserted gp41 via signal sequences such as Yxxφ or LL (Di Fiore & Gill, 1999; Fultz et al., 2001; Heilker et al., 1999; Sauter et al., 1996; Berlioz-Torrent et al., 1999). Detailed discussion of these are beyond the scope of this report, but we will touch on two issues: (a) we do not exclude the possibility that gp41 exists in the cell in both the conventional conformation and the new conformation proposed here, and (b) while the new conformation results in the inactivation of the first (most N-terminal) 719 Yxxφ signal by taking it outside the membrane, one of the suggested sequences for tm 3 (755–763) places the second 775 Yxxφ signal inside the membrane where it could be functional and permit recycling. Lastly, it may be significant that alterations to the C-terminal tail affect the conformation of both the gp41 ectodomain and of gp120 itself (Edwards et al., 2001, 2002; Spies et al., 1994; Vzorov & Compons, 2000).

What is the likely function of the gp41 minor ectodomain? One approach is to determine the specific virus life-cycle event that is inhibited when ERDRD-specific IgG neutralizes infectivity. So far we know that this antibody does not block attachment of virus to the target cell (Cleveland et al., 2000b), but does inhibit virus-mediated cell–cell fusion (L. Cheung & N. J. Dimmock, unpublished data). This fusion-inhibition had a similar dose-response to neutralization, suggesting cause and effect. Inhibition of fusion implies that the gp41 tail has a role in this process, but what this is remains to be determined. Much more experimentation is needed to reveal the complete structure and function of the gp41 C-terminal tail.

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