A Neutralizing Monoclonal Antibody Previously Mapped Exclusively on Human Immunodeficiency Virus Type 1 gp41 Recognizes an Epitope in p17 Sharing the Core Sequence IEEE

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We report here that a human immunodeficiency virus type 1 (HIV-1)-specific neutralizing monoclonal antibody (MAB 1575) mapped to the conserved putative intracellular region from amino acid residues 735 to 752 (735-752 region) of gp41 also recognizes a region in an extracellular portion of p17. Both epitopes have a core recognition sequence (IEEE) in a nonhomologous context. The IEEE motif found in HIV-1 p17 is located in a region known as HGP-30 (residues 86 to 115) which has been previously associated with virus neutralization, cytotoxic T lymphocyte activity, and mother-to-child transmission. An analysis of available gp41 and p17 sequences demonstrates that in these regions both IEEE sequences are highly conserved in different HIV-1 clades. The presence of the IEEE epitope in p17 allows us to explain some unexpected neutralizing characteristics of MAB 1575. In addition, the gp41 735-752 region has been previously reported both in intra- and extracellular locations. Our results suggest that the extracellular location was the result of cross-reactivity with p17.

A key element in understanding the events of human immunodeficiency virus type 1 (HIV-1) transmission and replication is the identification and characterization of epitopes which can induce neutralizing antibodies against a broad range of viral strains. These instruments can be used to elucidate the in vivo function of the different viral proteins. The importance of such knowledge for the development of effective vaccines is obvious. In recent years several studies have identified different regions of the envelope proteins of HIV-1 which are recognized by neutralizing antibodies (6, 15). The main antigenic determinants of the external glycoprotein gp120 have been identified in the V3 loop region (29) and the CD4 binding site (12), both of which are involved in the early stages of viral entry. In addition, two other neutralizing epitopes have been identified on the transmembrane envelope glycoprotein gp41, which is involved in the fusion of the viral envelope with the cellular membrane (15). One neutralizing epitope is located in the extracellular domain of gp41 (residues 647 to 671) (19, 20), while the other, rather surprisingly, is in a predicted intracellular location at residues 735 to 752 (735-752 region) (DRPEG IEEEGGERDRS) (14). Epitope mapping studies have shown that the two major epitopes of the 735-752 region are represented by the sequences IEEE and ERDRD (27). We have recently demonstrated that the detection of neutralizing antibodies against these two epitopes is dependent on their presentation in a conformation-specific manner (7). Although the in vivo significance of this region in HIV-1-positive patients is still controversial (6, 10, 24, 28), several studies have demonstrated that the 735-752 sequence is able to elicit high titers of neutralizing antibodies against different strains (8, 11, 17). Moreover, Binley et al. (3) have recently provided evidence that the immune response in HIV-1-infected patients against gp41 is directed predominantly against nonneutralizing regions. Therefore, further characterization of the 735-752 gp41

neutralizing motif may become very important for future work on subunit vaccines based on gp41. In addition, this epitope has been predicted to be a potential target for heterotypic neutralization of HIV-1 and HIV-2 strains (5). The possibility of inducing such heterotypic neutralization has been recently supported by the induction in rhesus macaques of cross-protection by HIV-1 recombinant poxvirus vaccines against HIV-2 infection (1). The lack of a plausible explanation for the neutralizing activity elicited by an intracellular domain of gp41 prompted us to look for alternative targets of the monoclonal antibodies (MAbs) in other HIV proteins.

The present study describes the identification of a conserved cross-reactive IEEE motif at residues 104 to 107 of HIV-1 p17 matrix protein. This protein is important in the early stages of the viral replication cycle and participates in the preintegration of the DNA complex into the nucleus of the host cells (21). The IEEE motif is found in a region of p17 known as HGP-30 (residues 86 to 115) which is of particular interest, since it was found to be the target of broadly neutralizing antibodies and cytotoxic T lymphocyte response against HIV-1 (2, 4, 22). Interestingly, the sequence from residues 98 to 108 (KEA LDKIEEEQ) was determined to be the HGP-30 major antibody binding site (4), and the KIEEEQN motif has recently been associated with mother-to-child transmission of this virus (21). In this study we demonstrate that a neutralizing antibody against the IEEE sequence of gp41 is capable of recognizing its corresponding sequence on p17. This finding explains some controversial results obtained by previous investigators on the intra- or extracellular location of the 735-752 epitope and on the neutralizing characteristics of a MAb directed against the IEEE motif in gp41 (9, 25, 27).

MATERIALS AND METHODS

Expression of recombinant and mutant p17 as a GST fusion protein. The coding sequence of HIV-1 isolate BH10 p17 (amino acids [aa] 1 to 132) (26) was amplified by PCR and cloned in the *Bam*HI and *Eco*RI sites of plasmid pGEX-3X (Pharmacia, Uppsala, Sweden), which allowed fusion to the N-terminal end of the enzyme glutathione *S*-transferase (GST). Primers and oligonucleotide sequences were based on the GenBank database entry HIVBH10, which corre-

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TABLE 1. Frequency distribution of the IEIE motif in p17 and gp41 proteins of different clades of HIV-1 and comparison of consensus flanking sequences

HIV-1 clades	p17 protein		gp41 protein	
	Consensus sequence (aa 99–116) ^a	No. of sequences with the IEIE motif/total no. of sequences (%)	Consensus sequence (aa 735–752) ^a	No. of sequences with the IEIE motif/total no. of sequences (%)
A	eAldki IEei qnKskgk??	2/34 (6)	dRper IEee GGeQdrdRS	7/21 (33)
B	EALdKi IEEE qnKskkkaq	29/32 (91)	drPegi ieee GGerdRdrS	58/69 (84)
C	EaLDki EEQ NK?Qqk??	5/7 (71)	DRlgr IEee GGEQDrdrS	10/12 (83)
D	EALeK EEEE qnKSKKk??	3/10 (30)	DRPegi EEEG GGEqGRdRS	8/11 (73)
E			DRpEr IEEG GGEQGRdRS	1/10 (10)
F	EALdK LEEE QNKSqQktq	0/5 (0)	DRPeg IEEG GGEqGkdRS	0/4 (0)
G	EALe EVEKa QknSQqk?Q	0/4 (0)	DRPEg IEEG GGEqdrdRS	0/3 (0)
H	EAL?K?? E ?QN??Q??--	0/2 (0)		
O	QAIQ LKEV -M?SRKS--	0/2 (0)	gTPGr TGeg GGeedRprW	0/3 (0)
U			DRPGR TEEG GGEEDNRS	0/1 (0)
CPZ	?A???? K ?????Q??T?	0/2 (0)	???? EI ? E ?GG??DR?R?	0/2 (0)

^a Consensus sequence alignments of residues 99 to 116 of p17 and of the corresponding residues 735 to 752 of gp41 in the different HIV-1 clades. This residue numbering refers to the HIV-1 IIB strain and may vary slightly in different viral strains due to deletions and insertions. Amino acids are indicated in single-letter code. Uppercase letters indicate 100% conservation, lowercase letters indicate $\geq 50\%$ conservation, and ? indicates $\leq 50\%$ conservation. The putative IEIE motif is in boldtype. Sequences were not available for all clades. Gaps introduced to optimize alignment are indicated (-).

sponds to the complete genome of HIV-1 isolate BH10. In addition, we created the E106G mutation in p17 from the BH10 strain by site-directed mutagenesis (13). Hence, the original IEIE sequence was mutated to IEGE. The resulting GST fusion proteins (GST-p17-IEIE and GST-p17-IEGE) were expressed in *Escherichia coli* as described previously (7) and purified by using glutathione Sepharose 4B agarose beads (Pharmacia).

FHV-L2gp41 and FHV-L2p17 recombinant proteins. The sequences of the gp41 735–752 neutralizing epitope (DRPEGIEEGGERDRDR) and the corresponding p17 residues (EALDKIEEEQNKSCKKA) were inserted in the L2 position on the surface of the Flock house virus (FHV) capsid protein (7). These proteins were produced in *E. coli* BL21 cells by using a pET expression system and purified as described by Buratti et al. (7). The resulting proteins were named L2gp41 and L2p17, respectively.

ELISA procedures and Western blot assays. Proteins (0.2 μ g/well; in 200 mM carbonate buffer, pH 9.6) and synthetic peptides (2 μ g/well; in 200 mM carbonate buffer, pH 9.6) were absorbed to a 96-well microplate (MaxiSorp; Nunc) by incubation overnight at 4°C. Synthetic peptides gp41pep (DRPEGIEEGGERDRDRS), p17pep (EALDKIEEEQNKSCKKA), and American BioTechnologies MN peptide (mnpep) (NKRKRIHIGPGRAFYTITKNIIGTIC) were synthesized in a Milligen 9050 peptide synthesizer (Millipore) and purified by high-pressure liquid chromatography. Peptides and proteins absorbed were washed three times with Tween-Tris-buffered saline (TTBS) (50 mM Tris [pH 7.5], 500 mM NaCl, and 0.1% Tween 20) (18) and incubated with the different MAb at room temperature (RT) for 1 h. In all assays, the different MAbs were used at the concentrations shown in the figure legends. After the proteins were washed three times with TTBS, a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (P447; Dako) at a dilution of 1:2,000 was applied to the wells for 1 h at RT. After the proteins were washed more three times with TTBS, the color was developed by using *ortho*-phenylenediamine dihydrochloride (Sigma-Fast; Sigma Chemical Co., St. Louis, Mo.). The reaction was stopped after 5 min by addition of 3 M sulfuric acid, and the optical density at 492 nm was measured. Western blotting was performed in phosphate-buffered saline (PBS) by using the same washing procedures as for the enzyme-linked immunosorbent assays (ELISAs), and the different MAbs were used at the dilutions given in the figure legends.

Peptide liquid-phase competition binding assays. The competition effects of the synthetic peptides on the binding of MAb 1575 (27) to L2gp41 and L2p17 recombinant proteins were determined as follows: 100- μ l portions of the diluted MAb 1575 were mixed with increasing amounts of competing peptide and added to the wells, and the plates were incubated for 1 h at RT. The bound MAb was then detected with the peroxidase-conjugated second antibody as described above.

Immunofluorescence of infected and uninfected H9 cells with MAbs 1575 and 2F5. H9 and HIV-infected H9/HTLV-IIIB cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (10 U/ml), and streptomycin (100 μ g/ml). HIV-infected and uninfected cells were washed twice with PBS and resuspended in PBS at 2×10^6 cells/ml; 0.1-ml samples were cytocentrifuged, fixed in a solution containing PBS, 5% formaldehyde, and 2% sucrose for 10 min at RT and washed three times with PBS containing 1% fetal calf serum. Immunofluorescence was performed with MAbs 1575 and 2F5 (19). Cells were incubated with 10 μ l of an antibody dilution (MAb 1575, 40 μ g/ml; MAb 2F5, 18 μ g/ml) at 37°C for 30 min and washed three times for 5 min each time with PBS before incubation with fluorescein isothiocyanate (FITC)-conju-

gated goat anti-mouse IgG, IgM, or IgA (for MAb 1575) or with FITC-conjugated affinity-purified sheep anti-human IgG, IgA, or IgM (for MAb 2F5) (The Binding Site, Birmingham, United Kingdom). After incubation for 30 min at 37°C, the slides were washed three times in PBS for 5 min, quickly rinsed with tap water, and mounted in 50% glycerol.

Computer sequence analysis. HIV-1 gp41 sequences were collected from the site <ftp://atlas.lanl.gov/pub/aids-db/ALIGN/ENV/> by downloading the file 1ENVpaln. HIV-1 p17 sequences were obtained from the site <ftp://atlas.lanl.gov/pub/aids-db/ALIGN/GAG/> by downloading the file 1GAGpaln.

RESULTS

Identification of IEIE motifs in HIV-1 genotypes. We compared the frequency of the IEIE motif in 98 available sequences of p17 belonging to different clades of HIV-1 with that observed in 136 sequences of gp41. The results are reported in Table 1, which shows that the IEIE motif in p17 is conserved to a high degree in clades B and C and to a lower degree in clade D. Likewise, the IEIE sequence in gp41 is very highly conserved in HIV-1 clades B, C, and D and is also present to a lesser extent in clade A. A comparison of the gp41 and p17 IEIE consensus flanking sequences (also reported in Table 1) shows that the two regions possess no additional homology. This finding suggests that different structural constraints on the two IEIE sequences might induce different conformations.

Recognition of the p17 IEIE motif in a recombinant p17 fusion protein. In order to investigate the ability of MAb 1575 to recognize the IEIE motif of p17, the whole coding region of p17 from HIV-1 BH10 isolate was expressed as a GST fusion protein (GST-p17-IEIE). In addition, we have also expressed a p17 fusion protein (GST-p17-IEGE) where the glutamic acid residue at position 106 was mutated to a glycine. Figure 1A shows that, while GST-p17-IEIE is highly reactive with MAb 1575, the mutation of the IEIE consensus sequence to IEGE completely abolished recognition. The specificity of binding of MAb 1575 to GST-p17-IEIE is shown in Fig. 1B, where it is clear that both p17pep and gp41pep can compete with GST-p17-IEIE. In addition, the results of Western blot analysis confirm that MAb 1575 was unable to react with the protein carrying the IEIE mutation (GST-p17-IEGE) (see Fig. 2A).

Recognition of the p17 IEIE motif in gp41 and p17. The reactivity of MAb 1575 with two synthetic peptides containing the p17 and gp41 IEIE sequences was then investigated (Fig. 1C). The results show that MAb 1575 is quite capable of

FIG. 1. (A) Reactivity of MAb 1575 with a recombinant p17 protein containing the IEEG sequence (GST-p17-IEEG) or the IEEE sequence (GST-p17-IEEE). (B) Competition effect of increasing concentrations of gp41 peptide (gp41pep) and p17 synthetic peptide (p17pep) on the binding of MAb 1575 to GST-p17-IEEE. (C) ELISA of the p17 synthetic peptide (p17pep), the gp41 synthetic peptide (gp41pep), and ABT MN peptide (mn pep) (a negative control) with decreasing concentrations of MAb 1575. (D) ELISA of the FHV hybrid proteins displaying either the p17 sequence (L2p17) or the gp41 sequence (L2gp41) and of the wild-type FHV protein (wt). (E and F) Competition effect of increasing concentrations of gp41 and p17 synthetic peptides on the binding of MAb 1575 to L2p17 (E) and L2gp41 (F). In panel C, the MAb 1575 dilution was 2 μg/ml, while in panel D, the dilution was 0.25 μg/ml. The datum points are the products of single ELISA wells, but similar data were obtained by repeating the experiment three times. OD₄₉₂, optical density at 492 nm.

reacting with the p17 IEEE motif, although with a lower reactivity than with the synthetic peptide containing the specific gp41 IEEE sequence. The ability of MAb 1575 to bind the p17 IEEE sequence was, however, more evident when this motif was displayed in a specific macromolecular structure by using the FHV capsid protein, which has already been demonstrated to improve the reactivity of these 735-752 epitopes (7) (Fig. 1D). The liquid-phase competition binding assays (Fig. 1E and F) are consistent with these findings. In order to clearly observe the competing effect of the peptides on both proteins, MAb 1575 was used at a dilution of 2 μg/ml when L2p17 was absorbed on the solid phase (Fig. 1E) and at a dilution of 0.25 μg/ml in the case of L2gp41 (Fig. 1F). The results show that

gp41pep is quite capable of competing well with both proteins (Fig. 1E and F), while p17pep is capable of competing only with the L2p17 recombinant protein (Fig. 1E). It must be noted, however, that as much as 50% inhibition of binding for the L2gp41 protein could be observed at higher p17pep concentrations (400 to 800 μg/ml) (data not shown).

In order to verify that MAb 1575 could also recognize the IEEE sequences present in gp41 and p17, we performed a LAV blot strip (Sanofi; Diagnostic Pasteur) assay (Fig. 2B, lane 1). The results show that reactivity can be seen with the gp160 precursor and with p17. No reactivity could be seen for the gp41 protein, but this lack of reactivity had already been noticed with HIV-1-positive sera and is probably due to the

FIG. 2. (A) Recognition of GST-p17-IEEE and GST-p17-IEGE with MAb 1575 (16.2 μ g/ml). Both the immunoblot (lower gel) and the Coomassie blue-stained gel of the purified proteins (upper gel) are shown. (B) Immunoblot of a detection strip (Sanofi; Diagnostic Pasteur) with MAb 1575 at a dilution of 16.2 μ g/ml (lane 1) together with the reference strip (lane 2). (C) Reactivity in the Western blot of the recombinant proteins L2p17 and L2gp41 and of the wild-type FHV capsomer protein (wt) with the IEEE-specific MAb 1575 (upper gel) and the ERDRD-specific MAb 1583 (lower gel). Each MAb was used at a dilution of 16.2 μ g/ml.

diffuse migration of this protein in the LAV strip because of its high glycosylation state (Fig. 2B, lane 2). A Western blot performed on a LAV strip with MAb 1583 (specific for the ERDRD epitope of the gp41 735-752 region) failed to recognize any specific protein (data not shown). However, this is not surprising, as the ERDRD epitope is highly conformational (7). Figure 2C shows the Western blot assays on purified L2gp41 and L2p17 proteins with MAbs 1575 and 1583. In keeping with the ELISA results, MAb 1575 was capable of recognizing both L2gp41 and L2p17, while MAb 1583 was capable of binding only to L2gp41. The lack of reactivity with MAb 1583 of L2p17 persisted even if the gel was overloaded with the protein (data not shown).

Immunofluorescence evidence for a different ectodomain of gp41 recognized by MAb 1575. Figure 3 shows the results of the observed immunofluorescence using MAb 1575 in HIV-1-infected and uninfected H9 cells. For a positive control (Fig. 3A), we used MAb 2F5 (19) which is specific for the ELDKWA ectodomain and is exposed on the surfaces of infected cells (25). An analogous result was obtained with MAb 1575 (Fig. 3B), indicating that this MAb recognizes an IEEE epitope also present on the surfaces of infected cells. Sattentau et al. (25) did not detect any increase in immunofluorescence with two MAbs (MAbs 1583 and 1908) specific for the ERDRD sequence of the 735-752 region (DRPEGIEEEGGERDRDRS) (27), showing that this epitope contiguous to IEEE is not expressed at the surfaces of infected cells. We have confirmed this observation, as immunofluorescence was not observed in our system when we repeated the experiment with the same MAbs (MAbs 1908 and 1583) under conditions identical to those in the experiment with MAb 1575 (data not shown). These data imply that the IEEE sequence of gp41, being very close to the ERDRD epitope (only two glycine residues away), is not exposed on the cell surface and that the specific reactivity observed is due to the presence of the homologous IEEE epitope in p17.

DISCUSSION

The observation that a conserved IEEE motif is present both on HIV-1 p17 and on gp41 is particularly interesting, since both sequences are present in regions that may be involved in neutralization of the virus and hence physiologically important for viral replication. The high degree of conservation of this motif observed in several viral genotypes in both p17 and gp41 clearly demonstrates that these sequences must play an important function in the life cycle of HIV-1.

Our results show that the two IEEE sequences share considerable antigenic cross-reactivity, although the homology in the flanking regions is very limited (Table 1 and Fig. 1). This cross-reactivity can explain some experimental discrepancies that have been described in previous studies of the 735-752 epitope (9, 25, 27).

Dagleish et al. (9) reported that three MAbs directed against a peptide containing the 735-752 sequence (DRPEGIEEEGGERDRDRS) were capable of giving an increase in immunofluorescence staining with infected CEM A3.01 cells. This suggested at first that this epitope was exposed on the surfaces of infected cells. However, a similar experiment performed by Sattentau et al. (25) using two MAbs directed against the ERDRD portion of this epitope (MAbs 1583 and 1908) failed to give a positive result. These data were consistent with the prediction that the 735-752 epitope should be located in the cytoplasmic tail of gp41 and therefore should not be available for antibody binding. The discrepancy between these two observations remained unclear. However, according

FIG. 3. Immunofluorescence visualization of binding of MAbs 2F5 (A) and 1575 (C) to infected H9 cells. Results of incubation with uninfected H9 cells are shown in the rightmost panels (B and D). Cells were counterstained with Evans blue, and bound antibody was visualized with an FITC-conjugated second antibody.

to our data, it may now be explained by presuming that the MAbs obtained by Dalglish et al. (9) (unfortunately unavailable to us) were directed against the IEEE portion of this epitope and that they were actually binding to the IEEE motif of p17 displayed on the surfaces of infected cells.

Vella et al. (27) incubated MAb 1575 and a poliovirus chimera carrying the 735-752 region of gp41 which contained the IEEE motif. The following selection of neutralization-resistant poliovirus escape mutants showed that one had mutated to IEIGE. This was unexpected, as IEIGE is the sequence found in the gp41 protein belonging to the RF strain of HIV-1. This strain, according to the data of Evans et al. (11), could be neutralized by MAb 1575 but only at low titers. Our results, obtained by mutating the IEEE sequence to IEIGE in a recombinant p17 fusion protein, clearly indicate that such a mutation completely abolishes recognition by MAb 1575. In light of this data, we can now explain this discrepancy by assuming that the neutralizing effect of MAb 1575 can also be mediated by its binding to the p17 protein of the RF strain which does carry the IEEE motif. This suggestion is also supported by the fact that neutralization could be seen only at low MAb titers, which is consistent with our observed lower affinity of MAb 1575 for the p17 IEEE motif. Similarly, Evans et al. (11) reported the ability of MAb 1575 to neutralize HIV-1 strain SF33, which has a IEEG motif in the 735-752 region. Unfortunately, no sequence data are available for p17 of SF33.

One question that still remains to be addressed is whether the IEEE epitope of p17 is actually exposed on the surfaces of infected cells. However, there is now a body of evidence that supports this possibility (23, 26). In particular, the work by Shang et al. (26) has demonstrated that four MAbs which bound to the surfaces of infected H9 cells were reactive against a p17 peptide (residues 86 to 115) which contained the IEEE motif. This finding has also been confirmed by the work of Liu et al. (16), who demonstrated that a peptide comprising resi-

dues 87 to 115 of p17 is exposed on the surfaces of infected cells.

However, it must be pointed out that the mechanism of neutralization of MAbs directed against the IEEE motifs of both gp41 and p17 still remains completely obscure and that very little evidence exists as to the physiological function of either of these two p17 and gp41 regions. Nevertheless, the unexpected finding that two IEEE sequences are highly conserved in the gp41 and p17 proteins in several HIV-1 clades implies that the possibility of cross-reactivity between these two epitopes should be considered when designing future experimental work and also opens further possible areas of study on the function and importance of these two regions. In addition, it makes the IEEE epitope an ideal target for generating cross-reactive neutralizing antibodies, which might be most advantageous in the development of broadly protective subunit vaccines.

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