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Biological and Immunological Properties of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein: Analysis of Proteins with Truncations and Deletions Expressed by Recombinant Vaccinia Viruses

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The effects of C-terminal and internal deletions on the synthesis, transport, biological properties, and antigenicity of the human immunodeficiency virus type 1 envelope protein were determined. A family of recombinant vaccinia viruses that express N-terminal overlapping *env* proteins of 204, 287, 393, 502 (full-length gp120), 635, 747, and 851 (full-length gp160) amino acids was constructed. All of the proteins were detected in intra- and extracellular forms which differed in the extent of glycosylation. The 747- and 851-amino-acid proteins were cleaved, were expressed on the surface of infected cells, and bound CD4. The 635-amino-acid *env* protein was cleaved inefficiently, and both the precursor and product were secreted, indicating absence of the transmembrane sequence. The 635- as well as the 502-amino-acid protein, which was also largely secreted, could still bind CD4. Unexpectedly, the 393-amino-acid protein was anchored in the plasma membrane, but neither it nor smaller proteins bound to soluble CD4. When amino acids at the gp120-gp41 junction were deleted, proteolytic cleavage of gp160 did not occur. Nevertheless, gp160 was inserted into the plasma membrane and bound soluble CD4. The predominant conserved B-cell epitopes were mapped to gp41 and the C terminus of gp120, whereas cytotoxic T-cell epitopes were distributed throughout the length of the glycoproteins.

The multifunctional envelope (*env*) glycoprotein of the human immunodeficiency viruses (HIV) plays a critical role in determining the cellular tropism of the viruses and is a prime target of the host humoral and cell-mediated immune defenses. This complex and highly mutable protein consists of interspersed variable and conserved domains (42, 47). After its biosynthesis, the polyprotein is glycosylated to form gp160, which is then assembled into oligomers and cleaved into gp120 and gp41 subunits (1, 11, 37, 40, 44). Although the gp120 molecules are completely externalized, some of them remain noncovalently associated with the transmembrane-spanning gp41 subunit. Both subunits are required for membrane fusion, gp120 in recognition of the cellular receptor CD4 and gp41 in interaction with the cellular membrane. A specific CD4-binding domain within the C terminus of gp120 has been identified by mutagenesis (25), and the hydrophobic N terminus of gp41 is required for fusion (23). The central conserved portion of gp120 is critical for infectivity but apparently does not participate directly in CD4 binding or syncytium formation (16, 48). Some mutations diminish the extent of cleavage or the association between gp120 and gp41 (23).

In view of its surface location, the *env* protein is accessible to antibody. Specific immunodominant epitopes in both gp120 and gp41 exist (4, 14, 36), and several regions in gp120 are targets for neutralizing antibodies (16, 17, 20, 27, 35, 41). In addition, cells expressing the *env* protein are lysed by major histocompatibility-restricted and -nonrestricted lymphocytes from HIV-infected individuals (21, 38, 45) as well as by antibody-dependent cell-mediated immune mechanisms (22, 28, 28). In the mouse, one cytotoxic T-cell (CTL) epitope within gp120 has been identified (3), but a systematic search for epitopes recognized by human CTL is yet to be described.

env proteins expressed in recombinant vaccinia virus-infected cells are glycosylated, processed into subunits, and transported to the cell surface; bind to CD4-bearing cells; mediate production of syncytia; and serve as CTL targets (6, 21, 26, 45). In this communication we describe the construction of a set of recombinant vaccinia viruses with mutant *env* genes that encode a full-length protein lacking the proteolytic cleavage site and six proteins with progressively larger C-terminal deletions. The effects of these mutations on the transport, cellular localization, biological properties, and antigenicity of the HIV-1 *env* protein are described. In addition, five $CD8^+$ *env*-specific CTL clones were generated from HIV-seropositive individuals and the set of recombinant vaccinia viruses was used to map the epitope-containing regions recognized by these clones.

MATERIALS AND METHODS

Construction of recombinant vaccinia viruses. The BH8 isolate of HIV-1 (39) was the source of the *env* gene used in all viruses. Oligonucleotide-directed mutagenesis of an M13 clone was used to generate a stop codon at the 3′ end of the gp120 coding sequence and in a separate reaction to eliminate 12 amino acids surrounding the proteolytic cleavage site between gp120 and gp41. These mutated *env* genes were then cloned into the plasmid vector pSC11 (5) to generate pPE8 (gp120 only) and pPE12 (deletion of cleavage site).

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The vector pSC11-ss, which contains a universal translation termination sequence downstream of the cloning site, was used to express the truncated genes. The *env* gene from which the two TTTTTNT vaccinia virus transcription termination sequences has been mutagenized (12) was the source of all truncated *env* genes. The set of truncated genes was constructed by digestion at unique restriction endonuclease sites within the open reading frame to create new 3′ ends as follows: pPE17 (*Bam*HI), pPE18 (*Hin*dIII), pPE20 (*Sca*I), pPE21 (*Pvu*II), and pPE22 (*Stu*I). Recombinant viruses were made by homologous recombination (3) and are named according to the plasmid from which they were derived.

Protein expression. For radioimmunoprecipitation (RIP), BSC1 cells were infected with 30 PFU of recombinant vaccinia virus per cell and metabolically labeled for 16 h with [^{35}S]methionine (Amersham). Cells were lysed and proteins were immunoprecipitated as previously described (11); the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide).

Digestions with endoglycosidase F (endo F) were performed on immunoprecipitated proteins as specified by the manufacturer (Du Pont).

For Western immunoblotting, infected cells were lysed 24 h postinfection and proteins were separated by SDS-PAGE (10% acrylamide), blotted to nitrocellulose, and incubated with antibody and then with ^{125}I-labeled protein A.

Immunofluorescence. CV1 cells were infected with 15 PFU of recombinant vaccinia virus per cell in eight-well tissue culture chambers (LabTek). After 16 h, cells were fixed with 3% paraformaldehyde for 20 min at room temperature, washed with phosphate-buffered saline containing 0.5% bovine serum albumin, and incubated with a 1:10 dilution of monoclonal antibody 902 (7) and subsequently with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (Boehringer Mannheim Biochemicals).

CD4 binding. Membrane-associated and secreted *env* proteins were metabolically labeled as described for RIP. Soluble CD4 was obtained from the medium of cells coinfected with recombinant vaccinia viruses VTF7-3 and vTMEB10 (32) and metabolically labeled with [^{35}S]methionine. *env* protein and excess soluble CD4 were incubated together for 3 h at 4°C and subsequently immunoprecipitated with excess OKT4 (Ortho Diagnostic Systems). In a separate reaction, an equivalent amount of *env* protein was immunoprecipitated with anti-*env* antibody. Proteins were analyzed by SDS-PAGE (10% acrylamide).

CTL clones and assay. Fresh peripheral blood mononuclear cells from HIV-1-seropositive donors were cloned at limiting dilution in the presence of phytohemagglutinin (0.2 μg/ml; Burroughs-Wellcome, Research Triangle Park, N.C.) and interleukin-2 (10% [vol/vol]; Electronucleonics, Silver Spring, Md.). Individual clones were screened for lytic activity against autologous lymphoblastoid cell lines infected with vSC8 (negative control), vPE7 (48), or vPE16. Clones demonstrating specific lysis against targets infected with vPE7 or vPE16 were expanded in the presence of interleukin-2 and examined for their ability to recognize lymphoblastoid cells lines infected with different *env* truncations as indicated above. Specific lysis was determined by using a 4-h ^{51}Cr release assay. Lymphoblastoid cell lines were infected with 2 PFU of recombinant vaccinia virus per cell for 10 to 14 h, labeled for 1.5 h with sodium chromate (Amersham), and added to the cloned effector cells in triplicate in the different ratios as indicated below.

RESULTS

Construction of recombinant vaccinia viruses that express mutated HIV-1 genes. To examine structure-function relationships of the HIV-1 glycoprotein and to locate B-cell and CTL epitopes therein, we constructed a collection of recombinant vaccinia viruses with mutated *env* genes. A nested set of six overlapping truncated genes with common 5′ ends was created by cutting the 3′ ends at unique restriction endonuclease sites located approximately 300 bp apart. These mutant genes, depicted in Fig. 1, were incorporated into recombinant vaccinia virus expression vectors.

The recombinant vaccinia virus expressing the complete *env* gene of HIV-1 isolate HTLV-IIIB (clone BH8), vPE16, has been described previously (12). Recombinant viruses vPE17 and vPE18 encode *env* proteins with progressively truncated forms of gp41; vPE8, vPE20, vPE21, and vPE22 encode gp120 and progressively shorter forms, respectively. The regions of *env* previously reported to be essential for transmembrane localization and CD4 binding as well as the sites of cleavage of the signal peptide and the gp120-gp41 boundary are indicated in relation to the *env* mutants (Fig. 1).

Another recombinant vaccinia virus, vPE12, containing an *env* gene with a deletion encompassing the site of proteolytic cleavage between gp120 and gp41, was constructed (Fig. 1). The codons for Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-Ala-Val, which contain primary and secondary consensus cleavage sites (18, 31, 44), were replaced with codons for Leu-Arg.

Characterization of the mutant proteins made by the recombinant vaccinia viruses. To examine the products of the natural and mutated *env* genes, we infected cells with the recombinant vaccinia viruses and metabolically labeled them with [^{35}S]methionine. *env*-specific proteins were immunoprecipitated with rabbit polyclonal antiserum to gp120 from the lysates and media of cells infected with each of the recombinant vaccinia viruses (Fig. 2A). The appearance of a 120-kDa band (vPE16, vPE17, and vPE18) indicated proteolytic cleavage at the gp120-gp41 boundary. Accurate size predictions for the truncated proteins could not be made because they are heavily glycosylated. Therefore, a portion of each immunoprecipitated sample was deglycosylated with endo F prior to PAGE (Fig. 2B). The molecular masses of the deglycosylated proteins, estimated from their mobility relative to size markers, corresponded to the predictions from the nucleotide sequence (Table 1).

In cells infected with vPE16 and expressing the wild-type *env* protein, gp160 was cleaved and some of the gp120 was shed into the medium (Fig. 2), as previously noted for HIV-infected cells as well as cells infected with vaccinia virus vectors (19, 33). The presence of gp41 in the cell lysate was demonstrated by using human serum (see below). Kinetic studies indicated that about 60% of the total *env* protein is cleaved to gp120 in this system and that more than half of that is shed (12a). The truncated *env* protein lacking the C-terminal 104 amino acids was cleaved to a similar extent as the full-length protein (Fig. 2, vPE17). The gp120 produced was not distinguishable from gp120 produced from wild-type protein (vPE16), indicating that glycosylation and cleavage were normal. When human serum was used, a truncated form of gp41 was detected (see below).

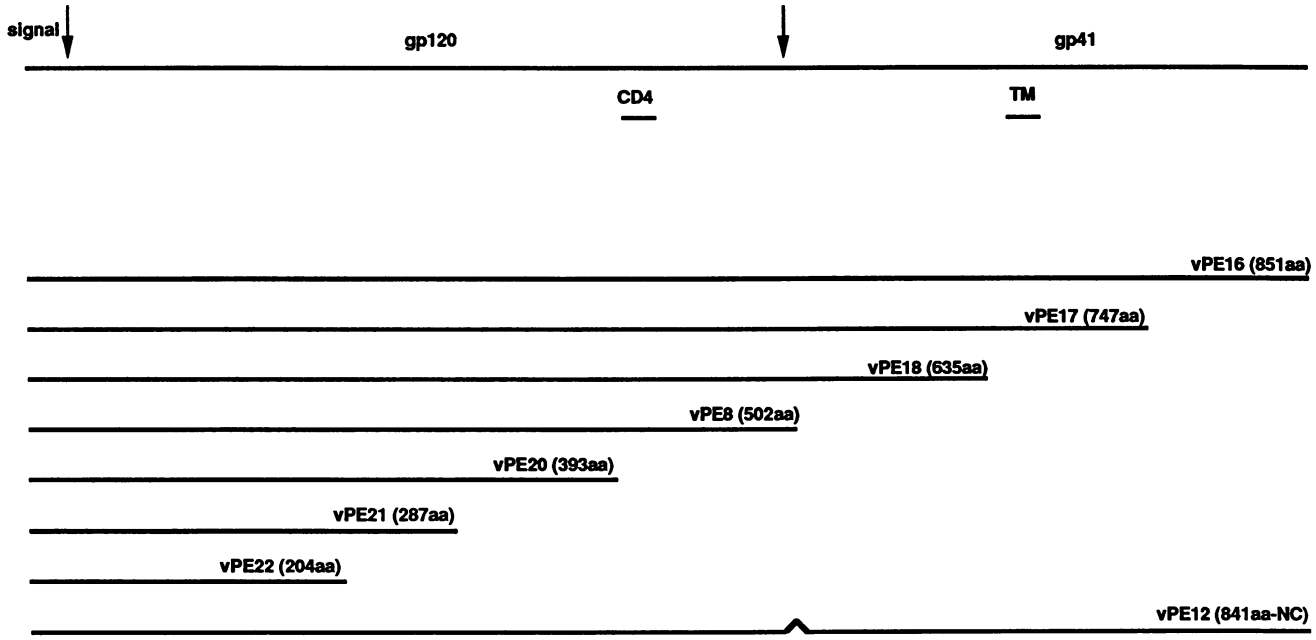

FIG. 1. Wild-type and mutant HIV-1 *env* proteins encoded by recombinant vaccinia viruses. The HIV-1 *env* protein is depicted at the top, with arrows indicating the locations of signal sequence and gp120-gp41 cleavage sites. The CD4-binding site (26) and hydrophobic transmembrane (TM) anchoring sequences are also shown. The overlapping lines represent the full-length and truncated *env* proteins expressed by the recombinant vaccinia viruses. The names of the viruses and lengths of the proteins in amino acids (aa) are indicated. The protein lacking the proteolytic processing site (841aa-NC) is also shown.

The recombinant virus vPE18 lacks the C-terminal 216 amino acids, which includes the highly hydrophobic putative transmembrane domain. This protein exhibited less cleavage to gp120, and both uncleaved and cleaved forms were secreted (Fig. 2, vPE18). The secretion of the uncleaved form supports the previous localization of the transmembrane region of the HIV-1 *env* protein (2, 13, 15). Despite the reduction in cleavage, the product that was cleaved still migrated with gp120 made from the wild-type protein.

The truncated *env* proteins of 502 and 393 amino acids were efficiently secreted (Fig. 2, vPE8 and vPE20). Anomalously, the still shorter polypeptides of 287 and 204 amino acids made by vPE21 and vPE22, respectively, were barely detected in the medium. (Autoradiographs of these lanes in

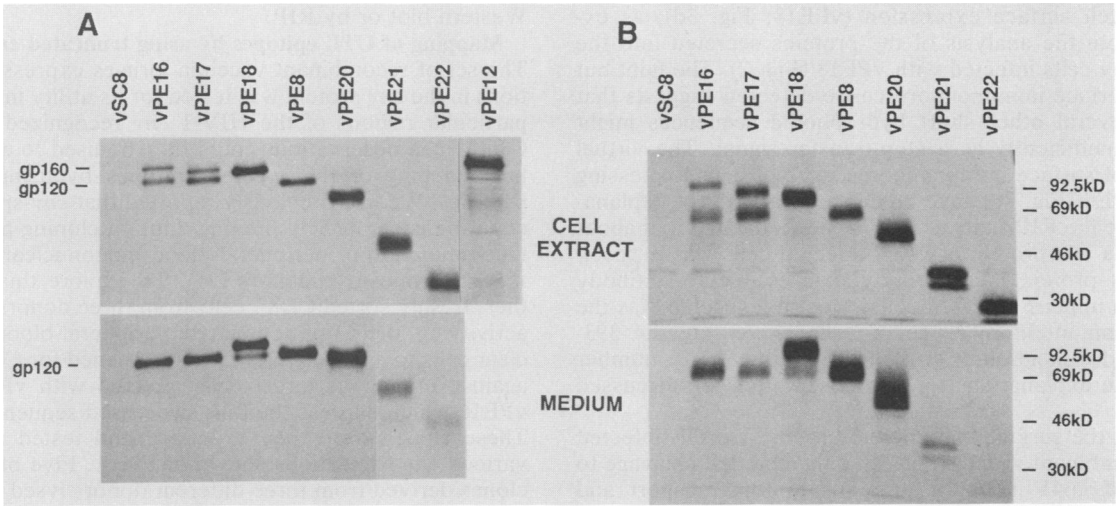

FIG. 2. Expression of wild-type and mutant HIV-1 *env* genes. (A) Metabolically labeled proteins from recombinant vaccinia virus-infected cell extracts or from the culture medium of infected cells were immunoprecipitated with rabbit polyclonal antisera to gp120 (PB33) and analyzed by SDS-PAGE (10% acrylamide). (B) Proteins were prepared as for panel A but were treated with endo F prior to electrophoresis. Molecular mass markers (Amersham) are shown on the right. vSC8 was used as a negative control; vPE8 encodes gp120; vPE16 is the wild type. Lengths of encoded proteins: vPE17, 747 amino acids; vPE18, 635 amino acids; vPE20, 393 amino acids; vPE21, 287 amino acids; vPE22, 204 amino acids; and vPE12, 841 amino acids (noncleaved).

TABLE 1. Molecular masses of vaccinia virus-encoded *env* proteins

Virus	Molecular mass (kDa)				
	Untreated		Endo F treated		Predicted[a]
	Cellular	Secreted	Cellular	Secreted	
vPE16	160/120	120	96/63	63	94/53
vPE17	150/120	120	85/63	63	81/53
vPE18	142	150/120	80	80/63	68/53
vPE8	110	120	63	63	53
vPE20	87	105	49	52	41
vPE21	60	72	35/33	35/33	29
vPE22	45	57	25	25	20

[a] Based on sequence.

Fig. 2 were overexposed.) Less efficient transport, cryptic stop-transfer sequences, and diminished stability in the medium are all possible explanations. After endo F digestion, the polypeptides appeared as doublets, probably as a result of incomplete deglycosylation since the smaller one of each set was closer to the predicted size.

As expected, the product of vPE12 was largely cell associated, was uncleaved, and comigrated with gp160 (Fig. 2). A small amount of cleavage, estimated to be less than 5% of that produced by vPE16, was observed and could have occurred at one of several lysines located just upstream from the deleted sequence or at the introduced arginine.

We noted, as have some previous workers (19, 24), that the uncleaved cell-associated proteins migrated slightly faster than the corresponding extracellular proteins. The differences were eliminated, however, by deglycosylation.

Cell surface expression of *env* proteins. The cell surface localization of the mutant *env* proteins was determined by immunofluorescence with a monoclonal antibody to gp120 (Fig. 3). Cells expressing *env* protein with the C-terminal 104 amino acids deleted (vPE17; Fig. 3c) were indistinguishable from those expressing the wild-type protein (vPE16; Fig. 3b). However, removal of 216 amino acids, including the hydrophobic region from amino acids 670 to 705, greatly reduced cell surface expression (vPE18; Fig. 3d), as expected from the analysis of the proteins secreted into the medium by cells infected with vPE18 (Fig. 2). The faint but definite surface immunofluorescence detected suggests that one of several other short hydrophobic sequences might function inefficiently as a stop-transfer signal. The virtual absence of surface immunofluorescence of cells expressing gp120 (vPE8; Fig. 3e) gave credence to the above explanation. Since the RIP analysis (Fig. 2) had indicated that about 30% of the *env* protein of vPE8-infected cells was intracellular, this protein is apparently inaccessible to antibody binding in unpermeabilized cells. Most unexpected was the surface immunofluorescence of cells expressing the 393-amino-acid truncation of gp120 (vPE20; Fig. 3f). A number of possible explanations for this observation are discussed below.

Finally, the surface immunofluorescence of cells infected with recombinant vPE12 (Fig. 3g) indicated that cleavage to gp120 and gp41 is not a prerequisite for transport and membrane anchoring. The less intense fluorescence of cells infected with vPE12 compared with cells infected with vPE16 may reflect less efficient transport to the cell surface.

CD4 binding of mutated *env* proteins. The ability of soluble CD4 to bind to mutated *env* proteins was measured by incubating metabolically labeled *env* proteins with excess soluble radioactively labeled CD4 and immunoprecipitating them with OKT4 monoclonal antibody. For comparison, a similar amount of each mutated *env* protein was immunoprecipitated with excess antibody to gp120. Each of the *env* proteins that contained at least the N-terminal 502 amino acids showed quantitative binding to CD4 (Fig. 4). The failure of proteins of 393 amino acids and shorter to bind CD4 was consistent with the previous localization of the CD4-binding domain between amino acids 397 and 439 (25). In addition, both uncleaved and cleaved proteins bound CD4 (Fig. 4, cell extract), suggesting that cleavage is not necessary to unmask the CD4-binding site. However, since unmasking may have resulted from the detergent extraction, we also examined CD4 binding to intact cells infected with vPE12, which has a deletion of the proteolytic cleavage site of gp160. CD4 binding was detected by a sandwich assay with a fluorescently labeled monoclonal antibody to CD4 (Fig. 5). The binding of the CD4 to cells that are deficient in cleavage of gp160 supports the contention that cleavage is not required.

Binding of antibodies to truncated *env* proteins. The usefulness of the nested set of truncated *env* proteins for defining antibody-binding regions was tested with a variety of sera. Mouse monoclonal antibody 902 was previously shown to bind gp120 (7). We found, by Western blotting, that all *env* proteins of 393 amino acids or longer reacted with this antibody (Fig. 6). Thus, deletion of the 96 amino acids between 287 and 393 destroyed the binding site.

Six different HIV-1-positive human sera were also examined by Western blotting. Two representative patterns are shown in Fig. 6. Five of the six sera, including serum 3192, reacted predominantly with conserved epitopes in gp41 and truncated gp41 as well as the uncleaved proteins containing these sequences. Although no reactivity with gp120 was detected by Western blotting, RIP analysis indicated that at least one of these sera (serum 3192) did react with full-length gp120 but not with any of the truncated gp120 molecules (data not shown). Serum 4312 was unique among the six in that it reacted with gp41 and truncations of that subunit as well as with gp120 on Western blots (Fig. 6). However, it did not react with any truncated forms of gp120 either by Western blot or by RIP.

Mapping of CTL epitopes by using truncated *env* proteins. The set of recombinant vaccinia viruses expressing truncations in the *env* protein was tested for its utility in identifying particular regions of the HIV-1 *env* recognized by cloned CTL. Such observations could then be used to assist in the fine mapping of the specific epitopes by using synthetic peptides. We had previously reported that *env*-specific CTL could be generated by limiting-dilution cloning and mitogenic stimulation of peripheral-blood mononuclear cells from HIV-1-seropositive donors (21). To explore this issue further, freshly sorted $CD8^+$ cells from three donors with CTL activity in their unfractionated peripheral-blood mononuclear cells to *env* were cloned and screened for CTL activity against autologous target cells infected with vPE7 (48) or vPE16 which express the fully processed sequences of *env*. These clones were then expanded and tested against the various *env* truncations shown in Fig. 1. Five of the $CD8^+$ clones derived from three different donors lysed target cells expressing different combinations of the *env* truncations, indicating that unique epitopes were being recognized (Fig. 7). In every case, the degree of lytic activity against target cells expressing truncated *env* proteins was comparable to the activity against the targets expressing the full-length *env* protein until all activity was lost. This pattern suggests that

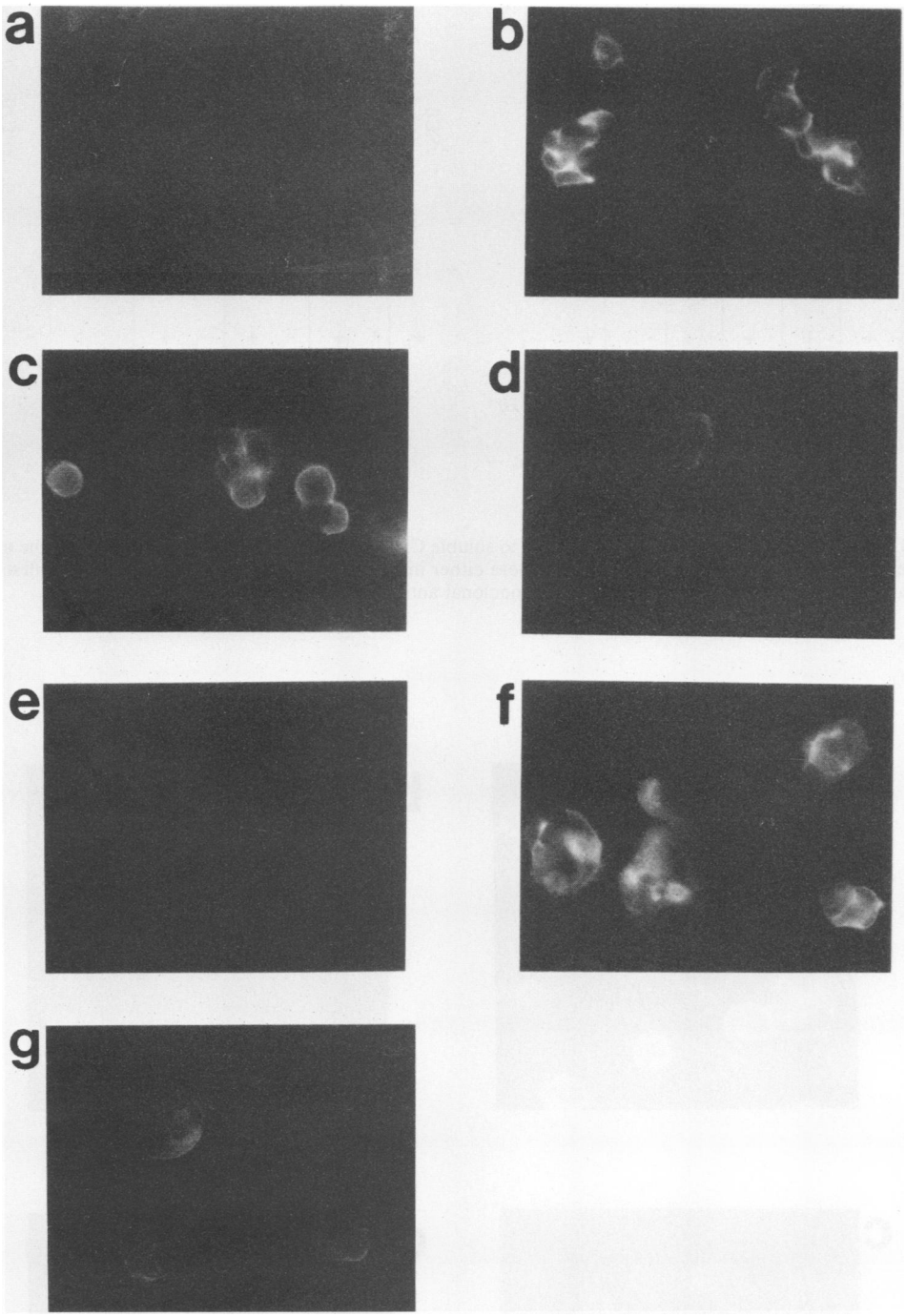

FIG. 3. Surface immunofluorescence of cells infected with recombinant vaccinia viruses. CV1 cells were infected with 10 PFU of vaccinia virus per cell, fixed with paraformaldehyde, and incubated with monoclonal antibody 902 followed by FITC-conjugated anti-mouse serum. (a) vSC8 (negative control); (b) vPE16; (c) vPE17; (d) vPE18; (e) vPE8; (f) vPE20; (g) vPE12.

the peptide segment forming a CTL epitope is located in the C-terminal segment of the shortest *env* protein that is recognized by a CTL clone (Fig. 8). It is of interest that the regions of the *env* protein recognized by these CTL include sites within gp120 and gp41 and that four of them are situated outside the only previously identified site for class I-restricted CTL activity (43). Surprisingly, one individual (patient SF, WR stage I) with good CTL responses to other HIV proteins (i.e., reverse transcriptase and *nef*) also had good CTL responses to more than one *env* epitope. The *env* CTL epitopes are probably located within conserved areas of the *env* protein because the protein expressed by these recombinant vaccinia viruses was derived from the IIIB sequence of HIV-1, a strain infrequently found in the North American population. All of the clones lysed target cells expressing *env* of the RF isolate (Fig. 7B) (data not shown), further support-

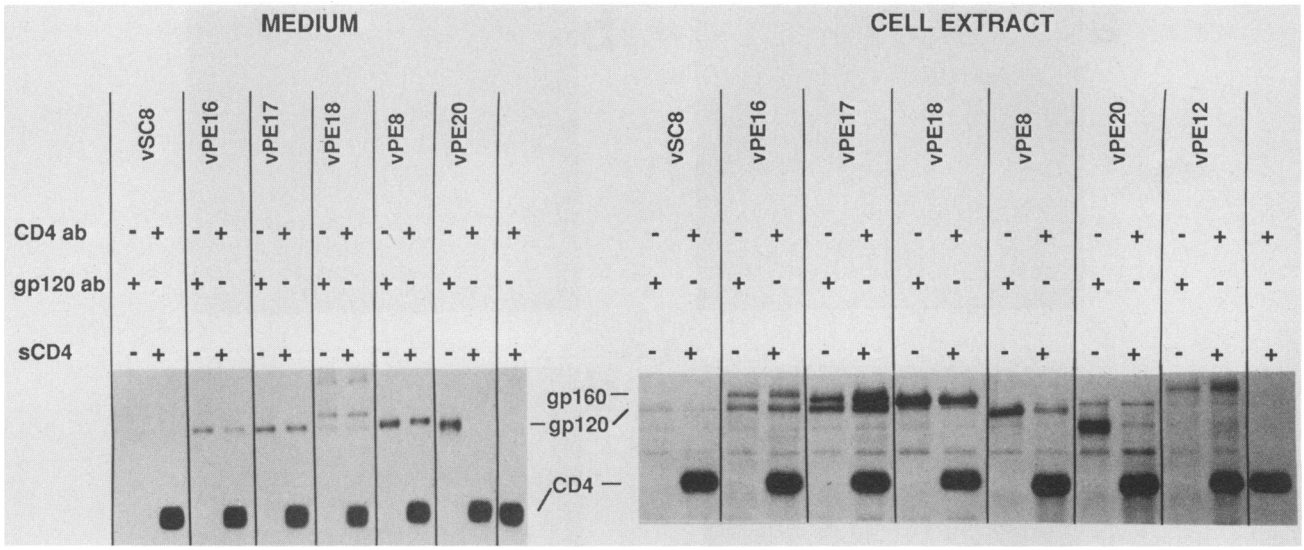

FIG. 4. Binding of wild-type and mutant HIV-1 *env* proteins to soluble CD4. Metabolically labeled proteins from the extracellular medium or from extracts of recombinant vaccinia virus-infected cells were either immunoprecipitated with anti-gp120 sera (first lane of each set) or coimmunoprecipitated with metabolically labeled CD4 and monoclonal antibody OKT4 (second lane of each set).

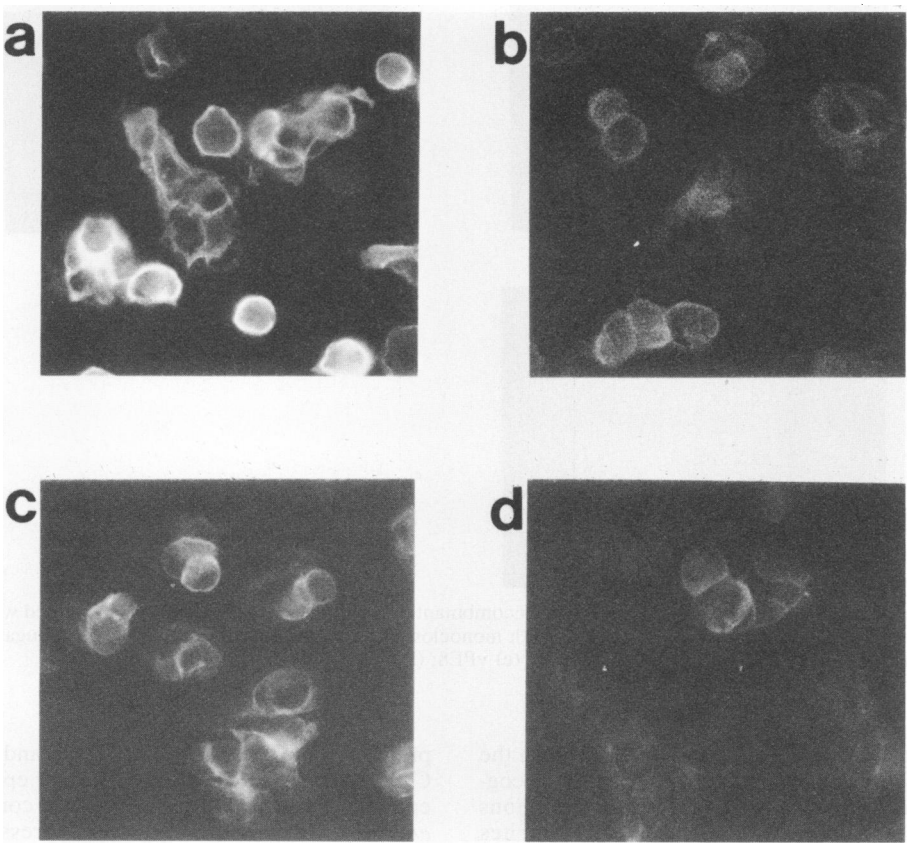

FIG. 5. Surface immunofluorescence of CD4 bound to cells infected with recombinant vaccinia viruses expressing HIV-1 *env* protein. Infected CV1 cells were incubated with monoclonal antibody to gp120 followed by FITC-conjugated anti-mouse serum or incubated with soluble CD4 followed by FITC-conjugated OKT4 antibody. (a) vPE16 plus monoclonal antibody 902; (b) vPE12 plus monoclonal antibody 902; (c) vPE16 plus soluble CD4 plus OKT4; (d) vPE12 plus soluble CD4 plus OKT4.

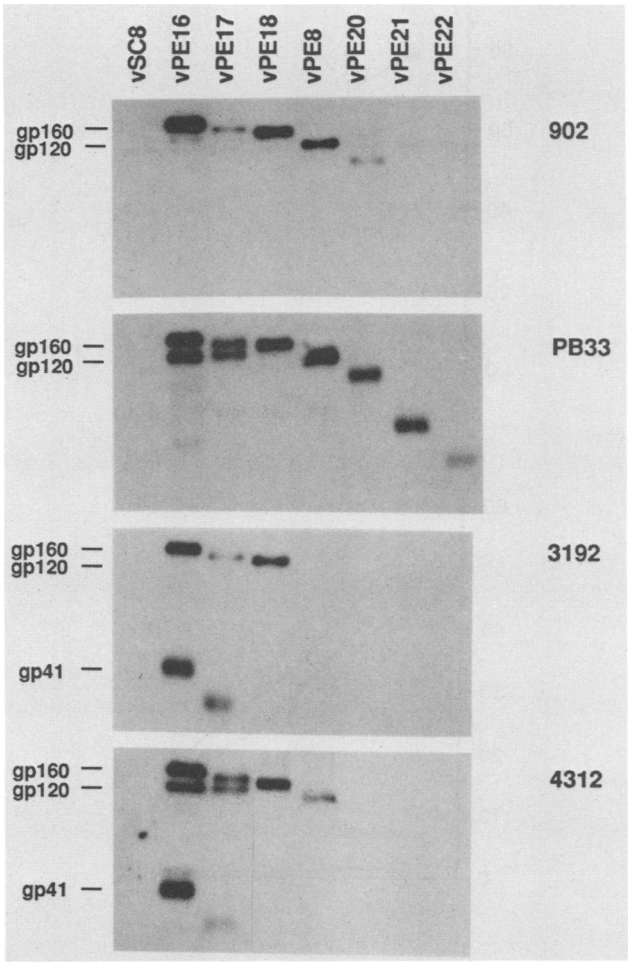

FIG. 6. Western blot of wild-type and mutant HIV-1 *env* proteins. Lysates from recombinant vaccinia virus-infected cells were separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose, and incubated with antisera and then with ^{125}I-labeled protein A. Panels: 902, monoclonal antibody to gp120 (7); PB33, rabbit polyclonal antisera to recombinant gp120 (Genentech); 3192 and 4312, HIV-1-positive human sera.

ing the notion of a group-specific response against conserved sequences.

DISCUSSION

We have constructed recombinant vaccinia viruses, containing a nested set of truncated HIV-1 *env* genes, and demonstrated their usefulness for functional and immunological studies. All of the proteins have the natural N-terminal signal peptide and, as a result, are translocated into the endoplasmic reticulum. The proteins are efficiently glycosylated and are transported to the cell surface or secreted, depending on the presence or absence of the membrane-spanning domain. These results and the retention of CD4 binding by the larger proteins suggest that the truncations have no global effect on protein folding. The structure of the truncated *env* proteins must be tested further, however, with a panel of conformational monoclonal antibodies.

The uncleaved intracellular form of the mutated *env* protein, in every case, had a lower apparent molecular mass than did the corresponding secreted molecule. This difference, however, was eliminated by deglycosylation. Similar observations were made with HIV-1 *env* proteins expressed in permanently transformed CHO cell lines (2, 24) and in cells infected with recombinant vaccinia viruses (19). These data suggest that the final steps in maturation occur concomitantly with or just prior to exist of the *env* protein from the cell. This interpretation agrees with kinetic studies which showed that transport of the influenza virus hemagglutinin between the endoplasmic reticulum and *trans*-Golgi is relatively slow but that secretion occurs rapidly after addition of sialic acid in the latter compartment (9a).

The functional properties of the truncated *env* proteins expressed by the recombinant vaccinia viruses were consistent with previously assigned locations of specific domains. Thus, deletion of amino acids 747 to 851, thought to be included in the cytoplasmic domain of gp41, did not impair transport, cleavage, or membrane anchorage (as shown here) or oligomerization (11). In addition, truncation of the cytoplasmic domain enhanced syncytium formation (data not shown) by an unknown mechanism.

The HIV-1 *env* protein has several stretches of hydrophobic amino acids. The transmembrane domain of gp41 is thought to reside between amino acids 670 and 705 (2, 13, 15). Our results support this interpretation since a mutant *env* protein truncated at amino acid 635 is largely secreted into the medium. Nevertheless, immunofluorescent-antibody binding suggested that some of the protein remained membrane associated. It is possible that the hydrophobic sequence between amino acids 375 and 395 functions as a weak stop-transfer sequence in truncated molecules produced by vPE18 and vPE20. Elimination of downstream sequences and exposure of this hydrophobic region at the C terminus could enhance its functioning as a stop-transfer sequence in vPE20. Alternatively, a cryptic site for glycosyl phosphatidylinositol anchor addition could be exposed by the truncation, or the secreted protein could be nonspecifically bound to the cell following secretion. Examination of the protein immunoprecipitated from extracts of cells infected with vPE20 shows the presence of two forms of *env* protein. The faster-migrating major band represents the incompletely glycosylated form, whereas the more slowly migrating minor band corresponds in size to the fully glycosylated secreted form. Since only terminally glycosylated molecules are secreted, one could hypothesize that the protein in this minor band observed by immunoprecipitation is responsible for the surface immunofluorescence. Alternatively, the incompletely glycosylated form may be selectively trapped in the plasma membrane.

In addition to truncation mutants, we constructed one *env* gene from which 12 codons including and flanking the proteolytic cleavage site sequence between gp120 and gp41 were deleted. The resulting protein folds and assembles normally (11), comigrates with gp160, and remains cell associated. Cells infected with this virus do not induce syncytia with CD4-bearing cells since cleavage to expose the amino terminus of gp41 does not occur. Since cells expressing this protein exhibit surface immunofluorescence, cleavage is not a prerequisite for transport. Using a recombinant vaccinia virus with a similar HIV-1 *env* mutation and serum from an HIV-1-infected human, Kieny et al. (18) also found cell surface expression. In that case, the immunofluorescence was much greater than that occurring in cells that expressed the wild-type protein, evidently because the latter shed gp120 extensively. Probably for a similar reason, they found that the recombinant vaccinia virus with the mutated

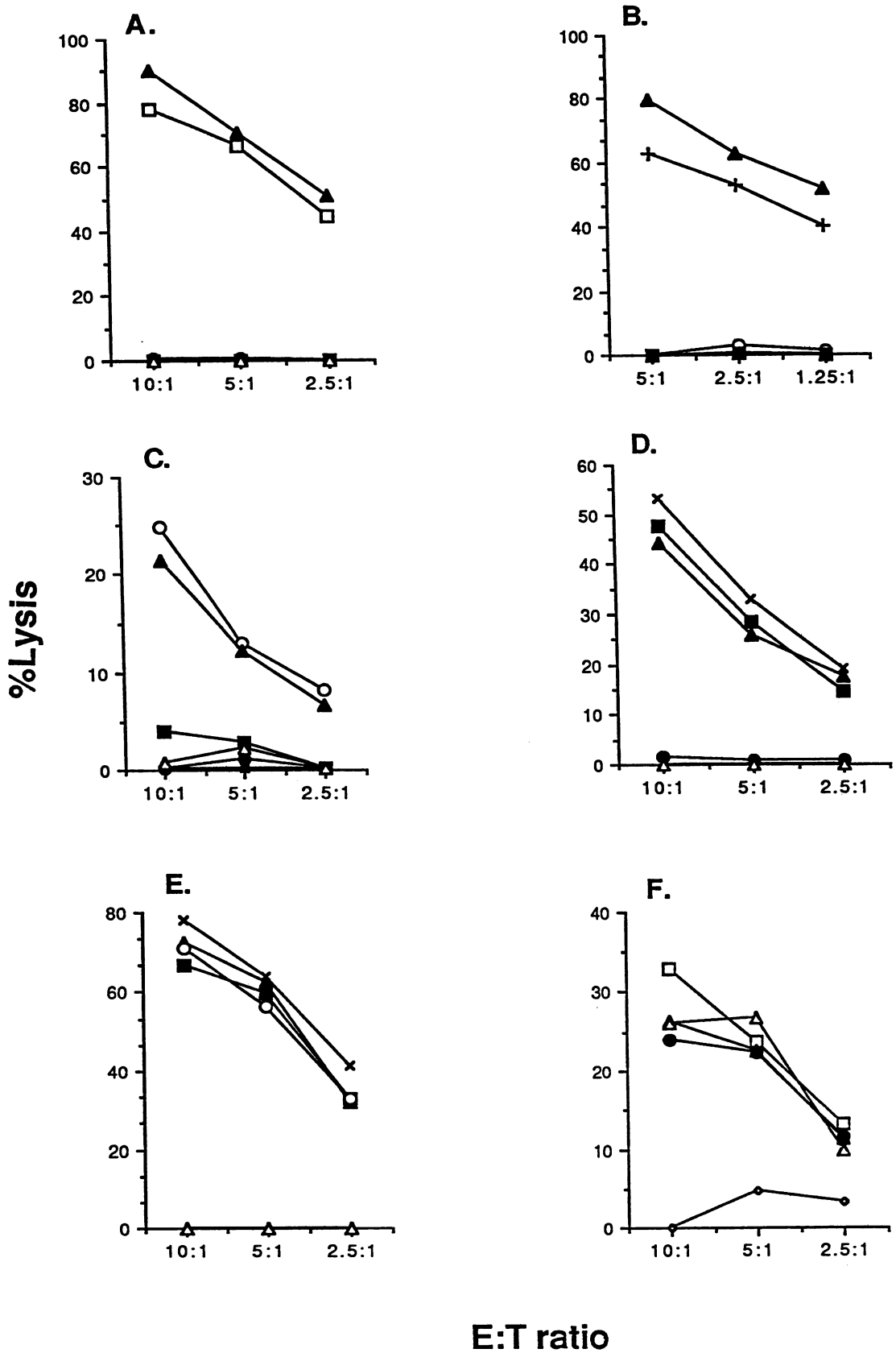

FIG. 7. Analysis of CTL clones by using recombinant vaccinia viruses. CTL clones derived from patients SF, BP, and AP were assayed for specific lytic activity as described in Materials and Methods. Percent specific lysis is shown on the *y* axis, and the effector/target (E:T) ratio is indicated on the *x* axis. Effectors tested were as follows: clone SF 1E91 (A and B), clone SF 1E85 (C), clone AP 2E345 (D), BP 4E12 (E), and SF 1E360 (F). Target cells used were as follows: vPE16 (▲), vPE18 (□), RFenv (+), vPE8 (○), vPE7 (×), vPE20 (■), vPE21 (●), vPE22 (△), and vSC8 (◇).

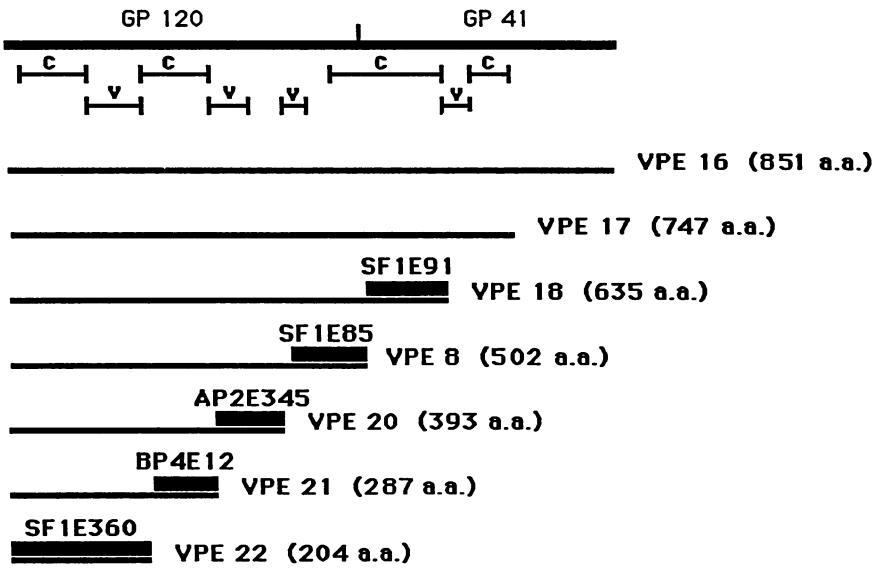

FIG. 8. Wild-type and truncated HIV-1 *env* proteins encoded by recombinant vaccinia viruses. The names of the viruses and the total number of amino acids (a.a.) of the HIV-1 *env* encoded by each truncation are shown to the right. Map locations recognized by the CTL clones are indicated by bold lines.

env was more immunogenic than recombinant virus with the parental *env*. The degree of gp120 shedding may be a function of the particular *env* gene used, since our recombinant expressing wild-type *env* did not shed as much gp120. In addition, our recombinant vaccinia viruses that expressed mutant and wild-type *env* were similarly immunogenic in mice (data not shown).

Mutations at several sites in gp120 prevent interactions with CD4 (8, 9, 10, 23), and changes between amino acids 397 and 439 may disrupt the binding site itself (25). We analyzed CD4 binding by two different methods. The first, which was carried out in solution, indicated that full-length gp160 and gp120 could bind CD4 but that a truncation between amino acids 393 and 502 led to loss of binding. This deletion brackets the previously determined CD4-binding region (25). The observation that gp160 binds CD4 suggests that cleavage is not necessary to unmask the site. However, since both gp120 and gp160 are present in cells expressing wild-type *env*, and the *env* protein oligomerizes, there was a possibility that only gp120 interacted directly with CD4. For this reason, the evidence for gp160 binding obtained with mutant vPE12, in which the cleavage site was deleted, was important. The possibility that the binding site was exposed by detergent extraction was eliminated by using a second CD4-binding assay, in which CD4 was shown by indirect immunofluorescence to bind to the surface of cells infected with vPE12.

The panel of recombinant vaccinia viruses was shown to be useful for rapidly mapping epitopes with one monoclonal antibody. Binding was eliminated when the 96-amino-acid segment between amino acids 287 and 393 of gp120 was removed. Examination of sera from six HIV-infected individuals indicated that the reactivity with gp41 was much greater than that to gp120. Further analysis indicated that the reactivity of gp120 was limited to the C-terminal portion. This distribution is consistent with the presence of more highly conserved sequences in these regions of *env* than in others (34). The evidence for proper glycosylation and transport of the truncated *env* proteins made by the recombinant vaccinia viruses enhances their potential usefulness in studying conformational epitopes, particularly when compared with *Escherichia coli* fusion proteins or synthetic peptides.

The mapping of CTL epitopes may be important in monitoring the immunological response of an individual to HIV infection as well as in designing synthetic vaccines. Owing to the size of the *env* protein, however, it is impractical to screen all CTL with activity against *env* expressed proteins with overlapping synthetic peptides representing the entire HIV-1 *env* gene. Therefore, the use of truncated *env* proteins expressed by recombinant vaccinia viruses is particularly valuable. A similar approach has been used to map epitopes within the smaller HIV-1 protein, *pol* (46). Our initial studies, presented here, validated this approach for the *env* protein. Unfortunately, the clonal *env*-specific $CD8^+$ lines used in these experiments could not be propagated in sufficient quantities to map the epitopes more specifically with peptides. The data obtained, however, give us some insight into the human CTL responses against *env*. For some proteins, such as the influenza virus matrix or hemagglutinin proteins, few epitopes are present that can induce CTL responses. Therefore, in these cases only a limited number of individuals may respond to that given epitope as a result of the restriction of that response to particular major histocompatibility complex class I molecules. In contrast, we have found that for HIV-1 *env*, a minimum of five epitopes can be recognized by $CD8^+$ CTL in conserved regions of the protein; this raises the possibility that cellular immunity can be generated in a vaccine engineered to express *env* in a more diverse subset of individuals of different human leukocyte antigen types. Further studies are needed to define the particular *env* epitopes and major histocompatibility complex class I proteins restricting these responses.

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