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The Human Immunodeficiency Virus Type 1 (HIV-1) CD4 Receptor and Its Central Role in Promotion of HIV-1 Infection

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INTRODUCTION

Infection of target cells by human immunodeficiency virus type 1 (HIV-1) is dependent on surface representation of cluster determinant 4 (CD4), which serves as a specific virus receptor (232, 276). High-affinity interactions between CD4 and the surface (SU) envelope glycoprotein of HIV-1 (gp120) initiate infection and viral entry (232, 296). HIV infection commonly leads to progressive loss of CD4-expressing cells, including CD4$^+$ T lymphocytes (26). This is correlated with the occurrence of opportunistic infections and development of AIDS (119).

In addition to peripheral blood lymphocytes, HIV-1 targets CD4$^+$ cells in the thymus, the primary site of CD4$^+$ T-cell selection (40, 437), and lymph nodes, a major HIV reservoir in asymptomatic infected individuals (346). HIV can induce cytopathic effects, including syncytium formation and cell death (270). It can also persistently infect CD4$^+$ cells both in vivo and in cell culture (193, 363, 405), leading to immunological dysregulation and a loss of immune competence in infected individuals (120).

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TABLE 1. Biological characteristics of known receptors for animal retroviruses

Virus[a]	Receptor name	Chromosome containing receptor		Biochemical characteristics of receptor	Cellular function of receptor	Structural homology of receptor
		Mouse	Human			
ALSV-A	*tv-a*			101–138-aa protein		LDL receptor
MuLV-E	*Rec1* (*Atrc1*)	5		622-aa protein	Amino acid transporter	Tea, H13
MuLV-A	*Ram1*	8	8	656-aa protein	Phosphate permease	GLVR-1
MuLV-P (MCFF)	*Rmc1*	1				*Sxv*
MuLV-X	*Sxv*	1				*Rmc1*
MMTV	*Mtvr1*	16				
M813 (asian MuLV)	*Rec2*	2				
FeLV-A				70-kDa protein		
BLV	BLVR-1			729-aa protein		
GALV FeLV-B MuLV-X*Caroli*	GLVR-1	2	2q	629-aa protein	Phosphate permease	pho4$^+$ permease
SRV-D FEV-C (RD114) BaEV-C REV			19q	60-kDa protein		
HTLV-I HTLV-II STLV	HTLV-R		17q	31–70-kDa proteins		
HIV-1 HIV-2 SIV	CD4	6	12	433-aa glycoprotein	T-lymphocyte coreceptor	IgG superfamily

[a] Abbreviations: ASLV, avian leukosis and sarcoma virus; MMTV, mouse mammary tumor virus; FeLV, feline leukemia virus; BLV, bovine leukemia virus; SRV, simian retrovirus; FEV, feline endogenous virus; BaEV, baboon endogenous virus; REV, reticuloendotheliosis virus; STLV, simian T-cell leukemia virus; MCFF, mink cell focus-forming virus; aa, amino acid; LDL, low-density lipoprotein.

This review deals with molecular mechanisms that underlie perturbation of CD4$^+$ cell function and maintenance of HIV infection; most such changes involve alterations of both expression and function of the CD4 molecule.

RETROVIRAL RECEPTORS

Receptors for Animal Retroviruses

Retroviral envelope proteins bind to specific receptor molecules expressed at the surface of susceptible cells. The identity of most retroviral receptors is still unknown. However, gene transfer technology and transfection studies have permitted the cloning and characterization of several molecules now known to function as cellular receptors for murine, avian, primate, and human retroviruses (Table 1).

One of the best characterized of these genes is EcoR, known to encode receptor activity for ecotropic murine leukemia viruses (MuLV-E) (8). The selective tropism of MuLV-E for mouse and rat cells allowed the isolation of the gene encoding EcoR after stable transfection of the nonpermissive human EJ cell line with mouse DNA. Transfected EJ clones were then challenged with a recombinant mouse sarcoma virus bearing an ecotropic envelope and the gene for resistance to the drug G418. A G418-resistant clone, susceptible to secondary infection with an ecotropic recombinant virus, was then probed against a mouse cDNA library to identify the full-length mRNA that encoded the virus receptor.

EcoR maps to the *Rec1* locus (now designated *Atrc1*) in the distal region of mouse chromosome 5 (247, 335), previously shown to be responsible for MuLV-E susceptibility (389). Expression of EcoR in *Xenopus* oocytes defined its normal function as a basic amino acid transporter (66, 229, 484). The selectivity and electrochemical properties of the EcoR transporter are identical to those of the previously identified y$^+$ cationic L-amino acid transporter, found at the plasma membrane of mammalian cells (359, 496, 497).

A human gene homologous to EcoR (H13) has been identified and mapped to chromosome 13 (514). Although H13 is unable to serve as a receptor for MuLV infection, it has a 87.6% amino acid homology with EcoR and shares 14 transmembrane-spanning regions with its mouse homolog (514). Since the highest diversity between H13 and EcoR is located within the third extracellular domain, this region appears to be critical for virus binding. Indeed, homolog-scanning mutagenesis showed that substitution of two amino acids in the third extracellular domain of H13 with those of EcoR rendered the human receptor functional for MuLV-E infection (515). The essential role of the third extracellular domain of EcoR in the permissiveness of MuLV infection was confirmed by studies of a unique receptor expressed by a fibroblastic cell line derived from the wild mouse species *Mus dunni* (MDTF). This receptor conferred only partial susceptibility to MuLV infection, since MDTF cells were resistant to Moloney murine leukemia virus (MoMuLV) infection (256). Comparison of amino acid sequences between the fully functional receptor expressed by NIH 3T3 cells and the restrictive receptor of MDTF cells showed an isoleucine-to-valine substitution at position 214 in the third extracellular domain, as well an additional glycine residue at position 236. Restoration of isoleucine 214 in the MDTF receptor conferred susceptibility to MoMuLV infection (114). However, the MDTF receptor could be rendered fully functional by treatment with the glycosylation inhibitor tunicamycin, introducing the notion that cell-specific N-linked

glycosylation could mediate susceptibility to MuLV infection (115).

The human gene conferring susceptibility to gibbon ape leukemia virus (GALV) has also been recently characterized. As with the strategy used to isolate the EcoR receptor for MuLV-E, the restricted tropism of GALV for human cells was used as a tool. Mouse NIH 3T3 cells, stably transfected with human DNA, were challenged with recombinant viruses pseudotyped with GALV that conferred drug resistance upon infection (333). The human gene encoding the GALV receptor was recovered, and cDNA derived from this gene was shown to confer susceptibility to GALV infection (333).

GLVR1 and its mouse homolog *glvr1* have been mapped to chromosome 2 in both species (5, 211). Interestingly, mouse chromosome 2 also contains the *Rec2* locus, which encodes a receptor used by the ecotropic Asian wild mouse virus M813 (366), suggesting a relationship between these receptors, since Asian wild mouse viruses appear to resemble GALV (268). The identification of a common receptor for GALV and amphotropic MuLV (MuLV-A) on E36 Chinese hamster cells (112) reinforces the possibility that related receptors can be used by widely different viruses. This notion is strengthened by the isolation and characterization of *Ram1*, the cellular receptor for MuLV-A (309).

The capacity of MuLV-A to infect cells over a wide range of species makes it difficult to find an indicator cell line resistant to primary infection that could become susceptible following transfer of a receptor gene (493). However, Chinese hamster ovary (CHO) cells are resistant to GALV, MoMuLV, and MuLV-A infection (456). Infection was abrogated either by tunicamycin-sensitive posttranslational modification of the receptor or by an inhibitory factor related to endogenous retroviral envelope molecules (27, 210, 517).

Somatic cell hybridization (among hamster and mouse or human cells) mapped the *Ram1* locus to mouse (146) and human (142) chromosome 8. Further genetic analysis with mouse cells showed that *Ram1* was located at the proximal region of chromosome 8 (275). Screening of a rat DNA library in CHO cells has shown that the *Ram1* locus confers susceptibility to MuLV (309). *Ram1* is structurally related to GLVR-1, although these two receptors only share 57 to 59% amino acid homology (309). However, *Ram1* was shown to be functionally similar to *GLVR-1*, since these two receptors are both sodium-dependent phosphate transporters (216).

A chicken gene, *tv-a*, has been shown to confer susceptibility to subgroup A avian leukosis and sarcoma viruses (517). The putative *tv-a* molecule apparently represents a novel class of retroviral receptors. Indeed, the quail homolog of *tv-a* also confers susceptibility to subgroup A avian leukosis and sarcoma viruses and has homology with the ligand-binding domain of the low-density lipoprotein receptor (27).

Our knowledge of most retroviral receptors is still limited to the chromosomal location of the loci responsible for susceptibility to infection. The loci responsible for susceptibility to mink cell focus-forming virus and xenoptropic MuLV (MuLV-X) were mapped to chromosome 1 (245, 246). Receptors for human T-lymphotropic leukemia virus I (HTLV-I) and simian D-type retroviruses localized to human chromosomes 17 and 19 (433, 434), and that for the mouse mammary tumor virus receptor mapped to mouse chromosome 16 (185). In other cases, cell surface proteins that specifically bind to retroviral envelopes have been isolated for human T-cell leukemia virus type I (144), simian retrovirus-1 (29), visna virus (86), and subgroup A feline leukemia virus (153) and await characterization. Finally, the cDNA encoding a functional receptor for bovine leukemia virus has recently been isolated. This gene encodes a 729-amino-acid membrane-spanning protein that confers susceptibility to bovine leukemia virus infection but has no known cellular function (20).

CD4 Is the Major Receptor for HIV-1 Infection

Early reports that HIV-1 infection in vivo was restricted to the $CD4^+$ subset of T lymphocytes (231) were followed by studies showing that anti-CD4 monoclonal antibodies (MAbs) could block both infection of $CD4^+$ target cells and subsequent formation of syncytia (85, 232). Immunoprecipitation with anti-gp120 antibodies revealed formation of complexes at the cell surface between the HIV-1 envelope glycoprotein and a 58-kDa molecule identified as CD4 (296). It was subsequently demonstrated that transfection of the CD4 gene into human cells and expression of CD4 at the cell surface conferred susceptibility to HIV-1 (276). However, mouse cells which express human CD4 can bind gp120 but are not infectable by HIV-1 (276), indicating that additional unidentified cellular factors, absent in cells of mouse origin, are required for this process (493).

In addition, alternative receptors may be used by HIV-1 to gain entry into cells that do not express CD4. The sphingolipid galactosyl ceramide has recently been shown to mediate infection of cell lines of both neural and epithelial origin (176, 511). The HIV-1 envelope protein gp120 is able to directly bind galactosyl ceramide at the cell surface (35) in a region distinct from the major binding site for CD4 (34). CD4 also serves as a receptor for the HIV-1-related viruses HIV-2 and simian immunodeficiency virus (SIV) (398). This is relevant since several viruses utilize members of the immunoglobulin (Ig) superfamily as cellular receptors (86, 307, 309).

ROLE OF THE CD4 CORECEPTOR IN T-CELL ACTIVATION

Structural Features of the CD4 Coreceptor

As depicted in Fig. 1, the CD4 molecule is a transmembrane glycoprotein of 58 kDa and consists of an extracellular region of 370 amino acids, a transmembrane region of 25 amino acids, and a cytoplasmic tail of 38 amino acids at the C-terminal end (277). The extracellular portion of CD4 is folded into four distinct domains designated D1 to D4 (69, 277, 278). The N-terminal D1 domain shares extensive structural and sequence homology with the variable region of Ig light chains. The other three domains are less closely related to Ig molecules at the level of primary structure but fold similarly to Ig family domains, confirming that CD4 is a member of the Ig-like superfamily (69, 277, 278, 508). Posttranslational modifications of CD4 include the formation of disulfide bonds which stabilize the D1, D2, and D4 domains and the addition of two N-linked glycans between D3 and D4 (277).

Crystallographic examination of the secondary structure of the D1 to D4 domains of CD4 has been limited by poor resolution, probably due to the flexible structure of the molecule between the D2 and D3 domains (87, 252). However, aspects of the predicted secondary structure of CD4 were recently confirmed by diffraction analysis of crystals containing the D1 and D2 domains (390, 486). These authors reported the resemblance of D1 and D2 to variable and constant Ig domains, respectively, and discussed the sharing of a β strand by D1 and D2, thus explaining the rigidity of the truncated molecule and its diffraction at high resolution.

Since extensive species conservation has been reported for amino acid sequences of the cytoplasmic tail of CD4, this

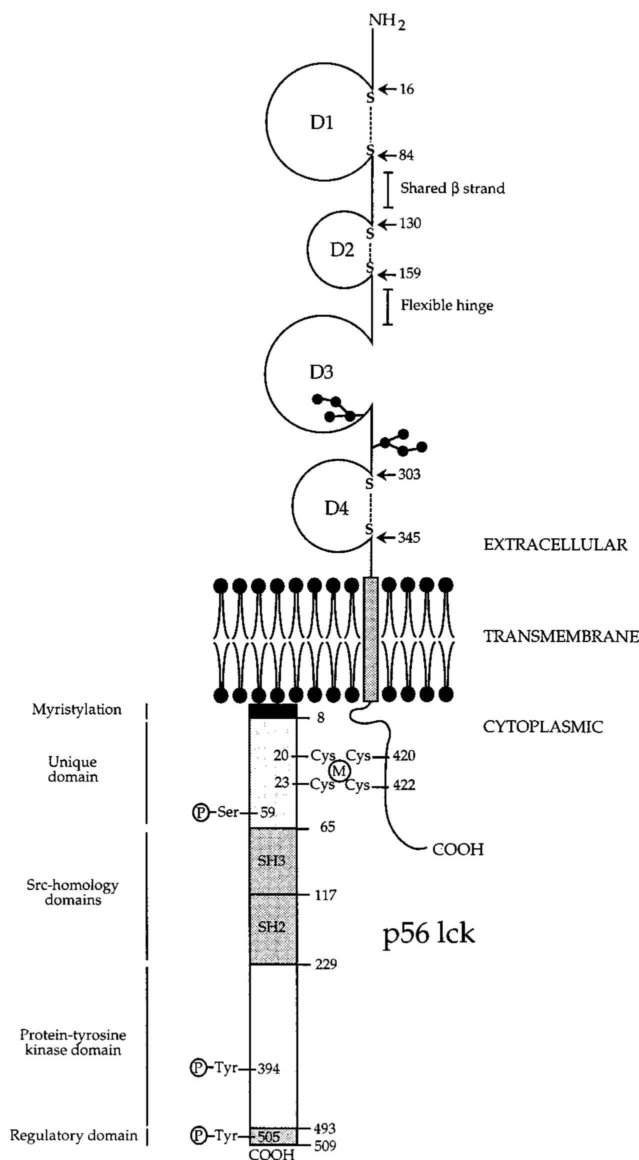

FIG. 1. Schematic representation of the CD4 and p56^{lck} molecules. The major structural features of the CD4 molecule are shown, including the D1-to-D4 immunoglobulin-like domains and the two N-glycosylation sites (●——●). The cysteine residues involved in disulfide bonds (S– – –S) are indicated by arrows together with their location in the amino acid sequence. The T-lymphocyte-specific protein tyrosine kinase p56^{lck} is also depicted. A pair of cysteine residues on each molecule mediates the association of Lck with the cytoplasmic tail of CD4 through the sharing of a metal ion (M). The site of tyrosine autophosphorylation (Tyr-394), the negative regulatory tyrosine at position 505, and the PKC-sensitive serine residue at position 59 are also shown.

region may be functionally important for CD4-mediated signal transduction across the plasma membrane in the context of T-cell activation.

Interactions of CD4 with Class II MHC Determinants

The subset of T lymphocytes that recognizes foreign antigens associated with self class II major histocompatibility complex (MHC) molecules is the predominant cell type that expresses surface CD4. Although CD4 is also present on monocytes/macrophages, B cells, and certain specialized cells of the central nervous system (137, 276, 278, 444), its immunological role has been elucidated only in the context of MHC class II-restricted T-cell activation. CD4 was originally described as a phenotypic marker of helper T lymphocytes (371). Although initial characterization attributed a helper/inducer function to $CD4^+$ T lymphocytes and a cytotoxic function to $CD8^+$ T cells, it quickly became apparent that clones of $CD4^+$ human T lymphocytes could also possess cytotoxic activity (303–305). As with helper T cells, these $CD4^+$ cytotoxic T lymphocytes displayed specificity for antigens expressed in association with class II MHC determinants (36).

The cytotoxic activity of $CD4^+$ T-cell clones can be inhibited by anti-CD4 MAbs, suggesting that CD4 contributes to T-cell function by direct binding to nonpolymorphic structures present on target cells (36). This was confirmed by studies showing that $CD4^+$ cytotoxic T-lymphocyte clones, whose T-cell receptors (TCR) had low affinity for MHC class II on target cells, were more susceptible to inhibitory effects of anti-CD4 MAbs than were cytotoxic T lymphocytes with high affinity (37). These data suggested that CD4 could act as a cell adhesion molecule stabilizing the interaction of low-affinity T cells with specific antigen-presenting cells (APC). Because of the specificity of $CD4^+$ T cells for MHC class II-expressing targets, it was postulated that CD4 could interact directly with MHC class II molecules.

However, the ability of the TCR on $CD4^+$ T cells to also bind to class II MHC molecules made it difficult to distinguish between the contribution of CD4 and the TCR in interactions with MHC class II determinants. Parallel studies with the mouse $CD4^+$ T-cell hybridoma 3DT 52.5, which expresses CD4 (L3T4), in conjunction with a TCR specific for mouse class I MHC molecules, have enabled this distinction to be made, since the TCR and CD4 are specific for different ligands. Activation of 3DT 52.5, as measured by production of interleukin-2, was enhanced when target cells expressing class I MHC molecules were further transfected to express class II MHC molecules (166). This stabilizing effect of CD4-class II MHC binding could be inhibited by either anti-CD4 or anti-MHC class II antibodies, indicating the specificity of such an interaction (165). After cloning of the human CD4 gene (277), the same model was used to detect the association of CD4 and human class II MHC molecules. Transfection of the human CD4 gene in a CD4 variant of 3DT 52.5 enhanced the ability of this cell line to produce interleukin-2 in response to HLA-DR (class II)-expressing but not H-2D^d (class I)-expressing target cells (145).

Although these studies demonstrated CD4–HLA-DR binding and its effect on T-cell activation, it remained unclear whether the TCR present on 3DT 52.5 cells mediated or contributed to this interaction or whether CD4 and class II MHC molecules interacted directly. Accordingly, when CV-1 fibroblasts were transfected to express large amounts of human CD4 and monitored for the ability to aggregate MHC class II^+ or MHC class II-B cell lines, adhesion occurred only with cells that expressed MHC class II antigens (104). Binding could be completely inhibited by a single anti-CD4 MAb or by a mixture of antibodies directed against all three major forms of human class II MHC, suggesting that CD4 binds to a nonpolymorphic region common to all class II MHC molecules. This was confirmed by showing that the CD4-binding site on both the mouse I-A and the human HLA-DR class II MHC was located within species-conserved residues of the β2 domain (50, 238).

Expression of CD4 is thus more closely associated with the MHC class II specificity of the TCR than with T-cell helper or cytotoxic function. Recent studies on cloned mouse and human $CD4^+$ T cells have shown that the effector function of these

cells is probably related to different patterns of cytokine production (90, 448).

CD4–T-Cell Receptor Interactions during T-Cell Activation

The sharing of a common ligand, i.e., MHC class II molecules, by CD4 and TCR suggests that direct interaction between these two molecules might occur during T-cell activation. By using MHC class II-restricted mouse T-cell clones that express both CD8 and CD4 and APC that express both class I and class II MHC molecules, it was shown that T-cell activation was 100 times more susceptible to inhibition by anti-CD4 than anti-CD8 MAbs (121). This suggested that anti-CD4 MAbs not only disrupted the binding to MHC class II molecules (which could have been compensated by CD8 binding to MHC class I) but also impaired the ability of CD4 to interact with the TCR. Double-immunofluorescence experiments confirmed that while the TCR and CD4 remained uniformly distributed at the surface of nonspecifically stimulated cloned murine helper T cells, specific antigen presentation induced a coclustering of CD4 and the TCR at the T-cell–APC contact site (251).

The presence of class II MHC molecules at the surface of the APC made it difficult to assess the contribution of the CD4-TCR interaction rather than the CD4-MHC interaction in the potency of T-cell activation. Further progress was potentiated by the use of anti-CD3 and anti-TCR MAbs as substitutes for APC in T-cell activation (219). Stimulation of T cells with high-affinity anti-TCR antibodies induced the association and cointernalization of CD4 and the TCR, and antibodies that did not induce such association were 30 to 100 times less potent at activating T cells (392). Furthermore, forcing CD4 to associate with the TCR by cross-linking anti-CD4 with anti-TCR MAbs allowed T-cell activation by anti-TCR antibodies that were not able to activate by themselves. Similar findings were obtained with anti-CD4 and anti-TCR antibodies cross-linked to Sepharose beads (15).

The ability of anti-TCR MAbs to activate T cells was shown to be related to both the TCR epitope recognized and the ability of the antibody to induce particular conformational changes within the TCR (384). Double-immunofluorescence studies showed that this conformational change favored the association of CD4 with the TCR and correlated with a 100-fold increase in T-cell activation (385).

The notion that antigen/MHC class II recognition induces the coclustering of CD4 and the TCR (16, 367, 376) has recently been challenged by studies of CD4-TCR interactions in resting T cells. Cell surface iodination followed by anti-CD4 immunoprecipitation revealed that the majority of cell surface CD4 was stably associated with the TCR complex through preferential interactions with the CD3 ζ subunit (452). Similar studies, performed with the human T-cell line HPB-ALL, demonstrated a physical interaction between the CD4-Lck complex and the ε and ζ subunits of CD3 (47). Thus, conformational changes in CD4, TCR, or both, following antigen presentation or binding of anti-CD3 MAb, may induce positional changes within the CD4-TCR complex that allow CD4 to interact with distinct TCR subunits in resting versus activated T cells.

Physical association of CD4 with the TCR during T-cell activation is thus an important process in enhancement of antigen presentation. Since increased T-cell responsiveness is dependent on recognition of the same class II MHC molecule by CD4 and the TCR (140), functional interactions between CD4 and the TCR probably serve to transform incomplete signals, independently generated by these two components, into a global activation signal. The mechanism by which CD4 participates in T-cell activation was therefore thought to involve the transduction of intracellular signals. Sequence conservation in the cytoplasmic domain of CD4 suggested that this region plays a significant role in this process. Enzymatic removal of the CD4 cytoplasmic tail permits one to distinguish between the adhesion enhancement and signal-transducing roles of CD4 (307, 428). CD4 that was deleted in this way was only able to potentiate antigen recognition and T-cell activation about 10 times less efficiently than native CD4. Thus, the signal transduction function of CD4 may assume greater importance than its adhesion role during T-cell activation.

Signal Transduction by the CD4-p56^{lck} Complex

A unifying explanation for the role of CD4 as a coreceptor for T-cell activation came from the finding that the p56^{lck} protein-tyrosine kinase is specifically associated with the cytoplasmic tail of CD4 (476) (Fig. 1). Lck is a member of the Src family of internal membrane protein tyrosine kinases and have been implicated in the control of T-cell activation and differentiation. Indeed, dysregulation and overexpression of p56^{lck} have been shown to promote tumorigenesis in both human and mouse cells (1, 217, 285, 286, 288). Structural features important for Lck function include (i) an amino-terminal myristylation required for membrane association; (ii) a Src-homology domain 3 (SH3) apparently required for association with the cytoskeleton; (iii) a catalytic domain containing the tyrosine 394 (Tyr-394) residue involved in in vitro autophosphorylation and positive regulation of Lck activity; (iv) a carboxy-terminal regulatory domain containing the tyrosine 505 (Tyr-505), phosphorylation of which inhibits Lck activity in vivo (285); and (v) an SH2 domain whose affinity for phosphotyrosine protein presumably modulates the interaction of Lck with its specific substrates.

The SH2 domain is also involved in intramolecular negative regulation of Lck, whereby phosphorylation of Tyr-505 induces its interaction with the phosphotyrosine-binding SH2 domain of the same molecule (424). The inferred conformational change might inactivate Lck by either masking the catalytic domain or impairing interaction with specific substrates containing phosphotyrosine amino acids (14). Another mode of regulation of p56^{lck} following T-cell stimulation involves the phosphorylation of serine residues, manifested through a mobility shift of p56^{lck} on sodium dodecyl sulfate (SDS)-polyacrylamide gels (287, 478). Although the exact role of serine phosphorylation in the regulation of p56^{lck} activity is not known, the serine residue at position 59 has been shown to be phosphorylated following TCR cross-linking or phorbol ester stimulation (489).

The amino acids involved in Lck interaction with the cytoplasmic tail of CD4 have been mapped to the 30 N-terminal residues of the tyrosine protein kinase (417). Substitution of this N-terminal domain by the corresponding domain of the related pp60$^{c\text{-}src}$ tyrosine kinase generated a hybrid molecule that was unable to bind CD4 (417). Among Src-related tyrosine protein kinases, Lck thus contains unique N-terminal residues that confer affinity for the cytoplasmic tail of CD4. Site-directed mutagenesis performed on the N-terminal sequence of Lck revealed the essential contribution of two cysteine residues at positions 20 and 23 for binding to CD4 (418, 470). These two amino acids interacted with a similar motif contributed by cysteines 420 and 422 in the cytoplasmic tail of CD4 (418, 470) (Fig. 1). Since the cysteine residues were not involved in intermolecular disulfide bonds, the interaction between CD4 and Lck was postulated to involve the sharing of a metal ion by the cysteine motifs of each molecule (418, 470).

The Lck protein kinase was shown to be expressed in both murine and human T cells but not B cells or monocytoid cells lines (388). Consistent with this tissue distribution, the zeta (ζ) component of the mouse TCR was shown to be a putative substrate of Lck phosphorylation (24, 477). Since tyrosine phosphorylation of the TCR appears essential for T-cell activation (209), the Lck protein kinase was assessed for its ability to enhance T-cell responsiveness. Antigen presentation by APC, cross-linking of CD4, and cross-linking of CD4 with the TCR were each shown to induce T-cell activation with the resulting phosphorylation of the ζ subunit of the TCR (2, 99, 477). Since this process was dependent on the association of Lck with the cytoplasmic tail of CD4, it was concluded that Lck was the intracellular component responsible for CD4-positive signal transduction (154). The inability of CD4 molecules, unable to bind Lck, to enhance anti-TCR antibody-mediated T-cell activation was shown to result from impaired association of CD4 with the TCR complex (76). In addition, the CD4-Lck complex appears to restrict T-cell activation through TCR αβ (but not CD3) stimulation when not closely associated with the T-cell antigen receptor (181). The Lck protein tyrosine kinase thus appears to be essential for both CD4 subcellular localization and initiation of signal transduction events leading to T-cell activation following MHC class II-restricted antigen presentation.

The ζ subunit may not be a unique substrate of Lck, as alternative substrates of Lck that may be phosphorylated after T-cell activation include a group of mitogen-activated proteins, the Ras GTPase-activating protein, and phospholipase C-γ1 (13, 118, 490). Lck interactions with different substrates may occur at different stages of T-cell activation or development.

CD45, a specific surface antigen of hematopoietic cells that exhibit protein tyrosine phosphatase activity, has recently been involved in regulation of Lck activity (reviewed in reference 240). Activation of p56lck was reduced in a murine T-lymphoma cell line lacking CD45 compared with that in $CD45^+$ counterparts (323). This correlated with increased phosphorylation of Lck at the inhibitory Tyr-505 residue (339) and inactivation of the enzyme by intramolecular interactions between Tyr-505 and the SH2 domain (424). Cross-linking of CD45 and CD4 confirmed that CD45 could directly dephosphorylate the Lck Tyr-505, leading to increased kinase activity (340). Association of CD45 with Lck was shown to occur between 72 and 96 h after T-cell activation (312). The CD45 phosphatase thus not only appears to restore and maintain a constant pool of active Lck but also may protect the cell from redundant antigenic stimulation, by delaying Lck reactivation until late after initial stimulation. Since $CD4^+$ memory cells but not naive human lymphocytes express an isoform of CD45 that is permanently associated with CD4 (98), it is possible that these memory cells express a high-activity Lck protein kinase that is permanently dephosphorylated at position 505 and hence able to be fully activated after CD4 cross-linking and Lck phosphorylation at tyrosine 394. This unique structure of the CD4-Lck-CD45 complex may play a role in the efficiency of memory T-cell activation. These results also show how the expression of different isoforms of CD45, which associate with CD4 or other components involved in T-cell activation, can modulate the delivery of activation signals in cells of different species and origin (67).

The counterpart of CD45 appears to be a cytoplasmic protein tyrosine kinase, termed $p50^{csk}$, which downregulates Lck catalytic activity by specific phosphorylation of the Tyr-505 residue. Since $p50^{csk}$ possesses a phosphotyrosine-binding SH2 domain but no myristylation signal, it may be recruited by the phosphorylated Tyr-394 residue of activated Lck (31). Although details of the putative $p56^{lck}$-$p50^{csk}$ interaction remain to be clarified, the role of Csk in the negative regulation of Src family kinases and T-cell activation is now well established (64, 324, 451).

CD4 Can Transduce Inhibitory Signals to T Cells

In addition to its role in transducing a positive signal to T cells upon cross-linking with the TCR, an intriguing feature of CD4 is its ability to transduce a negative signal when cross-linked in the absence of the TCR-CD3 complex. TCR-independent ligation of CD4, by cross-linked MAbs, was shown to inhibit the proliferation and interleukin-2 production signals delivered by mitogenic lectins (23, 464, 488). Anti-CD4 MAbs were also shown to inhibit $CD4^+$ cytotoxic T-lymphocyte-mediated cell killing induced by anti-CD3 MAbs or mitogenic lectins (129). These studies were performed in the absence of APC, ruling out the possibility that the inhibitory effect of the anti-CD4 MAbs was due to impaired antigen recognition. In addition, the use of bivalent and monovalent Fab fragments of anti-CD4 MAbs provided a system able to distinguish between whether the inhibitory effect of anti-CD4 MAbs was due to physical cross-linking of CD4 or whether it was due to induction of steric hindrance impairing the proper association of CD4 with the TCR (175). Fab fragments of anti-CD4 antibodies, unable to cross-link CD4, were devoid of ability to inhibit lectin-induced proliferation but retained the capacity to block T-cell activation mediated by anti-CD3 MAbs. In contrast, bivalent anti-CD4 antibodies were able to deliver a negative proliferation signal to T cells. Cross-linking of CD3 with CD4 enhanced the mobilization of cytoplasmic free calcium, $[Ca^{2+}]_i$ in comparison with stimulation by anti-CD3 MAbs alone; however, independent aggregation of these components inhibited the mobilization of $[Ca^{2+}]_i$ (262).

Thus, a single transduction pathway, triggered by cross-linking, can account for both CD4 positive and negative signaling. This transduction pathway may involve activation of $p56^{lck}$, since cross-linking of CD4 but not TCR (typically generating a "negative-signaling" response) enhanced the catalytic activity of Lck despite an apparent increase in the phosphorylation of the negative regulatory Tyr-505 residue (475). The latter may represent a late event, occurring after the initial activation of Lck, since cross-linking of CD4 was also reported to induce Lck activation concomitant with increased phosphorylation of the positive regulatory Tyr-394 residue (273). Although the pattern and kinetics of Lck phosphorylation may depend on both cell type and methods of T-cell activation, these results indicate that cross-linking of CD4, in the presence or absence of TCR-CD3 cross-linking, induces the activation of Lck catalytic activity.

Delivery of a proliferative rather than an inhibitory signal thus depends on the physical approximation of the CD4-Lck activated complex with relevant substrates within the TCR-CD3 complex. This could involve either the migration of CD4-Lck close to the activated TCR or, more probably, conformational changes in preformed CD4-TCR complexes that would allow interactions of Lck with the activating CD3 ζ-chain substrate (452, 475). Failure to induce necessary conformational changes within the TCR would impair Lck association with specific substrate or, alternatively, expose an inhibitory substrate within the TCR, leading to negative signaling. CD4 negative signaling may be involved in nonspecific binding of APC to T cells, potentially leading to apoptosis when followed by antigenic stimulation (39, 201, 292, 329).

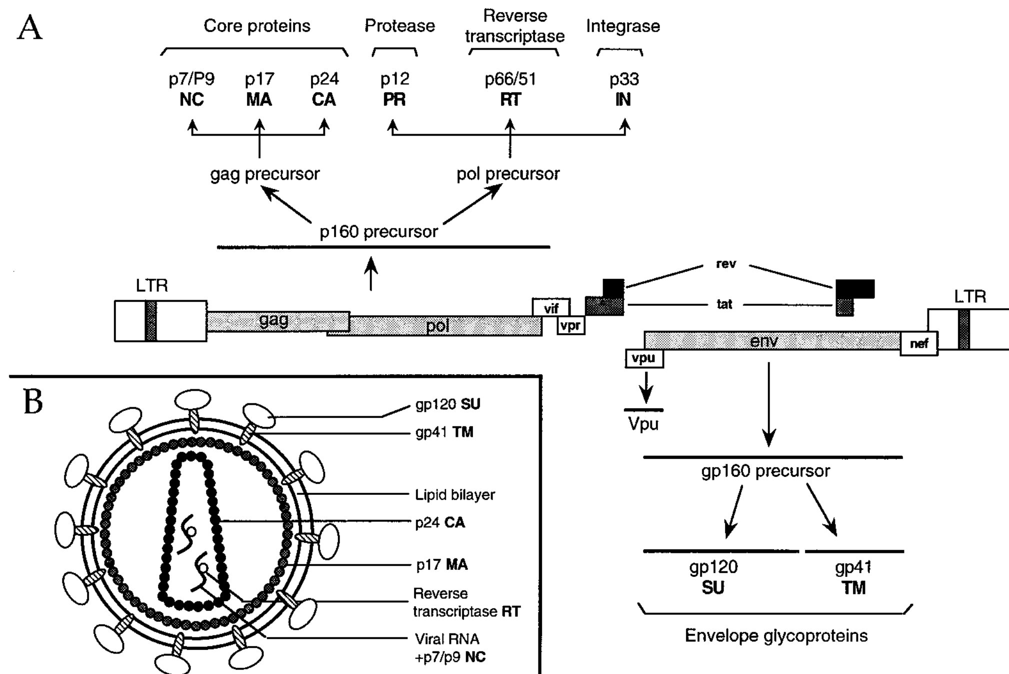

FIG. 2. Structure of the HIV-1 genome and viral particle. (A) Genetic map of HIV-1 proviral DNA and expression of regulatory and structural viral proteins. (B) Schematic representation of HIV-1 after assembly and budding. LTR, long terminal repeat.

STRUCTURE AND MATURATION OF HIV-1 ENVELOPE GLYCOPROTEINS

Biosynthesis of the gp160 Polyprotein Precursor

The HIV-1 envelope glycoprotein is encoded by a bicistronic mRNA containing the *env* open reading frame (ORF) just downstream of the *vpu* ORF (414) (Fig. 2A). The bypassing of ribosomes and initiation at a downstream *env* AUG occur as a consequence of leaky scanning of the weak initiation sequences of *vpu* to yield a 90-kDa protein that is glycosylated in the endoplasmic reticulum (ER) (415). The gp160 Env glycoprotein precursor is then generated by addition of about 31 *N*-asparagine-linked, mannose-rich oligosaccharide chains (9). Oligopeptide mapping and amino acid sequencing reveal that gp160 is cleaved to yield gp120 and gp41 as mature envelope proteins (Fig. 2A) (101, 379). Infection of cells with recombinant gp160-expressing vaccinia viruses and pulse-chase and surface immunofluorescence protocols have established a precursor-product relationship between gp160 and gp120/gp41 and the presence of these proteins at the cell surface (56). External viral gp120 (SU) is responsible for binding of the CD4 receptor (296), while transmembrane gp41 (TM) anchors gp120 through noncovalent interactions and mediates membrane fusion with target cells (139, 140, 244, 431) (Fig. 2B).

Endoproteolytic Cleavage of gp160

Processing of gp160 occurs in the *cis* or medial compartment of the Golgi apparatus together with the trimming by α-mannosidase I of mannose-rich oligosaccharide chains (96, 439). Research has identified two putative tryptic-like cleavage sites in gp160, one of which, an Arg-508–Gln–Lys–Arg-511 site, is located at the C terminus of gp120. This sequence is highly conserved among retroviruses and is preferentially used to generate gp120 and gp41 (294). When this site was replaced by a chymotryptic-like equivalent, cleavage of gp160 was blocked, although no effect on either synthesis or cell surface representation of gp160 was found (294). Cells that expressed such a cleavage-deficient form of gp160 could no longer fuse with $CD4^+$ cells, suggesting that the fusion activity of gp41 is dependent on prior cleavage of the envelope precursor. An arginine-to-threonine substitution at position 511 generated cell surface expression of cleavage-deficient forms of gp160; such cells were unable to fuse with $CD4^+$ cells despite their ability to bind CD4 (171).

The other tryptic-like site, 500-Lys–Arg–Lys–Arg–Arg-504, contributes basic amino acid residues that may potentiate proteolysis at the first site. Endoproteolytic cleavage of gp160 at the 500 to 504 site is responsible for 10 to 20% of total gp160 cleavage (125); however, the gp41 thus produced may suffer from impaired formation of oligomers and be biologically inactive (125). Cleavage of gp160 may also be abolished by substitution of a glycine or serine for a pair of highly conserved cysteine residues in the extracellular domain of the gp41 subunit (88, 454). Impaired recognition by a cellular protease of the cleavage site and/or improper transport of gp160 to the cleavage site could result from disruption of this disulfide-bonded loop. Proteolytic cleavage is thus highly dependent on the proper folding, oligomerization, and glycosylation of gp160. In certain cell types, cleavage of gp160 is very inefficient and most of the envelope precursor is relocalized to a lysosomal compartment where degradation occurs (498).

Furin, a subtilisin-like eucaryotic endoprotease, has been identified as a cellular enzyme responsible for gp160 cleavage. Exposure of CV-1 cells to recombinant vaccinia viruses, expressing both gp160 and human furin but not gp160 alone, was necessary for processing of gp160. Specific inhibitors of furin blocked production of gp120 and gp41 (173). Although furin has been confirmed to process gp160 in a baculovirus system (322), this enzyme may not be solely responsible for gp160 cleavage in mammalian systems. Indeed, gp160 was recently shown to be efficiently processed in a human colon carcinoma

cell line that was furin defective (334). Moreover, the isolation of a novel ER-associated protease that cleaves gp160 in Molt-4 T-lymphocytic cells suggests that the proteases involved in gp160 maturation could be cell type specific (227).

Glycosylation and Trimming

The envelope protein gp120 has a complex oligosaccharidic structure consisting mainly of asparagine-linked structures (see reference 368 for a recent review). Compounds that inhibit the glucose and mannose trimming of the precursor oligosaccharides Glc_3-Man_9-$(GlcNac)_2$ of gp160 have been used to study the effects of glycosylation on gp160 transport and cleavage. Such trimming is required for conversion of some of the mannose-rich oligosaccharide structure found in gp160 to the more complex-type oligosaccharides present in mature gp120 (9, 152). First, three glucose residues are cleaved by glucosidases I and II in the ER; this step can be inhibited by 1-deoxynojirimycin and castanospermine. Subsequently, mannosidases I and II cleave a total of six mannoses from the Man_9 moiety in the Golgi, preceding addition of complex-type sugars. Mannosidases I and II can be specifically antagonized by 2-deoxymannojirimycin and swainsonine, respectively.

Several studies have suggested a direct correlation between the trimming state of gp160 and the generation of gp120 and gp41. When the glycosylation inhibitor tunicamycin was used to treat chronically infected Molt-3 cells, the result was an accumulation of the envelope precursor in the form of a nonglycosylated 80-kDa molecule that did not undergo further processing (344). Other studies that used different oligosaccharide-trimming inhibitors concluded that gp160 cleavage depended on glucosidase I but not mannosidases I and II. Therefore, gp160 transport from the ER to the Golgi, where cleavage takes place, may follow removal by glucosidase I of glucose residues in the high-mannose structure.

Addition of complex oligosaccharide chains may occur only after gp160 cleavage (96, 439). gp120 and gp41 are ultimately brought to the trans-Golgi, where they are terminally processed by addition of complex carbohydrates (439). Virion surface gp120 contains a mixture of complex oligosaccharides and oligomannosidic chains. High-pressure liquid chromatography and enzymatic digestion showed that these complex sugar moieties, containing glucose and sialic acid, represent around 40% of total glycans (152). The gp120 contains 24 N-linked sugar moieties, of which 13 are complex oligosaccharides (266). Mutagenesis experiments showed that only five N-glycosylation sites in the N-terminal half of the gp120 molecule are important for viral infectivity (263). Binding to CD4 is unaffected by removal of these glycosylation sites. However, a mutation at asparagine 267, important for infectivity (263), altered gp120 secondary structure, thus impairing complex interactions among its distinct domains (506).

Oligomeric Structure of the Envelope Glycoproteins of HIV-1

The ER is the site of generation of gp160 dimers and possibly of tetramers and is also important in gp160 transport to its cleavage site (111). Immunoblot analysis of HIV-1 lysates, probed with anti-gp41 antibodies, revealed the multimeric nature of gp160. Trimers and tetramers of gp41, resistant to dissociation by SDS, migrated at relative molecular sizes of 120 and 160 kDa, respectively, distinct from gp160 and gp120 (357). Sucrose gradient analysis of cell-associated envelope glycoproteins showed that monomeric gp160 rapidly formed homodimers (and higher-order structures, e.g., trimers or tetramers) that were stabilized in a noncovalent manner by amino acids 68 to 129 in the extracellular segment of gp41 (108, 110). Brefeldin A could not block the formation of oligomers, which were formed within 30 min of synthesis of gp160, indicating that oligomerization had probably occurred in the ER (111). The gp160 oligomeric structure was also unaffected by proteolytic cleavage, implying that mature gp120 and gp41 envelope glycoproteins may also be organized on the virion surface as oligomers (492). This may be important in infectiousness and cell fusion by promoting multimeric binding of HIV-1 to CD4 receptors (109).

The vesicular stomatitis virus G protein (249), influenza virus hemagglutinin (151), Rous sarcoma virus Pr95 (116), and the HIV-1 related HIV-2 and SIV (102, 373) are other viral systems in which envelope precursors must oligomerize as a step preceding transport to the Golgi and proteolytic cleavage. Misfolding of HIV-1 gp160 in the ER generates oligomers that can no longer undergo proteolysis as a result of stabilization by intermolecular disulfide bonds (341). Substitution of a leucine for aspartic acid in the second conserved domain of gp120 yielded a similar result, because misfolding and formation of covalently linked oligomers prevented export of gp160 from the ER (500). Therefore, proper folding, glycosylation, oligosaccharidic trimming, and oligomerization of gp160 are all required for cleavage to gp120 and gp41 to occur.

MOLECULAR CHARACTERIZATION OF CD4-gp120 INTERACTIONS

Role of Oligosaccharides in CD4-gp120 Interactions

Infection of target cells was blocked by binding of mannose-specific lectins onto gp120 (270, 380). Thus, oligosaccharides on gp120 may be involved in CD4 interactions, consistent with results showing that deglycosylation of native and/or recombinant gp120 blocked such associations (123, 290). Inhibition of binding may, however, have been due to structural changes within gp120 rather than the absence of glycans, since relevant gp120 deglycosylation was enacted in the presence of denaturing agents such as SDS. Only a 2- to 20-fold decrease in affinity for CD4 was caused by enzymatic deglycosylation of recombinant or native gp120 in the absence of SDS (124, 126), consistent with the notion that the oligosaccharide coat surrounding gp120 may protect the molecule from antibody-mediated immune recognition by hiding relevant gp120 amino acid sequences, rather than through CD4 binding. Enzymatic removal of the two N-linked oligosaccharide chains of CD4 by enzymatic digestion had no effect on gp120 binding (124). Moreover, normal binding to gp120 was observed with a truncated, nonglycosylated form of CD4 that contained the gp120-binding site; this suggests that this process can occur independently of glycosylation (57). Therefore, both three-dimensional folding and the primary structures of both gp120 and CD4 appear key in defining mutual affinities.

Binding Site for gp120 on CD4

Proteolytic fragmentation of purified soluble CD4 showed that peptides consisting of the intact disulfide-linked D1 domain of CD4 were able to bind gp120 (374). Only a single D1 domain-related synthetic peptide (i.e., residues 25 to 28) of many D1 and D2 peptides tested (Fig. 1) was able to inhibit HIV-1-induced cell fusion in a dose-dependent manner (202). Epitope mapping of CD4 substitution mutants with MAbs showed that a small region, containing amino acids 41 to 52, was involved in gp120 binding (397). Despite extensive homology with human CD4, mouse CD4 (L3T4) cannot bind gp120.

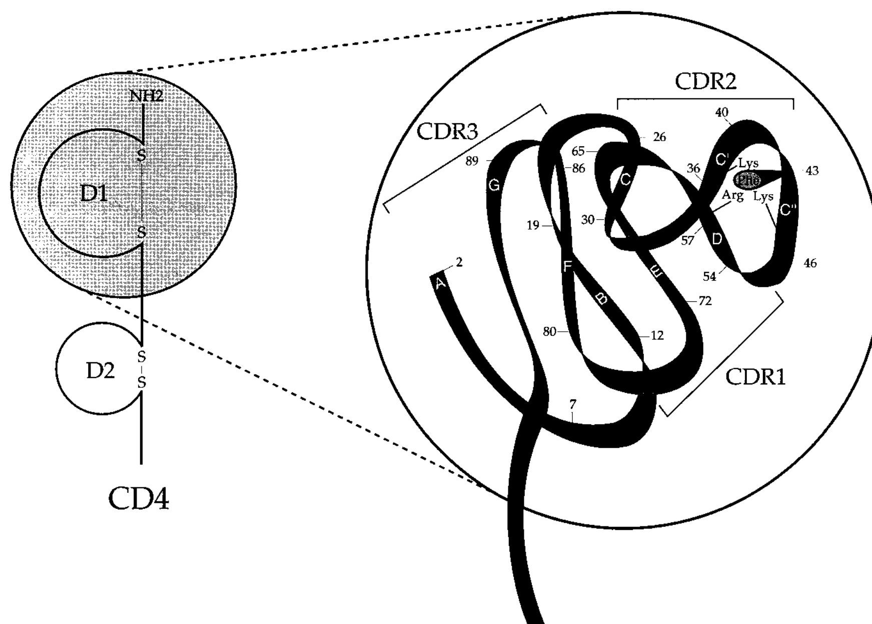

FIG. 3. Atomic structure of the CD4 D1 domain and gp120-binding site. A three-dimensional ribbon representation of the CD4 immunoglobulin-like D1 domain is presented. The β-strand structures are represented according to the crystal structure of the D1 and D2 domains of CD4 and identified by the letters A to G (390, 486). Numbers refer to the positions of amino acid residues at the boundary of each β-strand. The β-strands of the CD4 D1 domain form β-sheets analogous to the Ig complementarity-determining regions and are termed CDR1 to CDR3. The CDR1 to CDR3 regions are formed by β-strands BED, CC′C″, and AFG, respectively. Most of the amino acid residues involved in gp120 binding are clustered in the CDR2 domain. In particular, a phenylalanine residue at position 43 forms a hydrophobic pocket surrounded by hydrophilic residues at positions 35, 46, and 59.

Substitution of nonconserved amino acids from the L3T4 D1 domain onto human CD4 showed that residues 38 to 57, located in a region analogous to an Ig light-chain variable domain (CDR2), were indispensable for gp120 binding (70, 255) (see Fig. 4). Studies, in which CD4 mutants were selected through loss of reactivity with MAbs against different CD4 epitopes, showed that binding of gp120, as measured by syncytium formation, required residues 42 to 49 in the CDR2 domain (354). The opportunity for finer analysis of the CD4-binding site was provided by the resolution of the atomic structure of the D1 and D2 domains of CD4 (390, 486). Mutagenesis was performed on residues in close proximity in the C, C′, C″, and D strands, encompassing part of the CDR1 and all of the CDR2 regions (Fig. 3). The results showed that four charged residues (Lys-29, Lys-35, Lys-46, and Arg-59) and one hydrophobic phenylalanine at position 43 were essential for gp120 binding (63, 315, 469). These five amino acids are predicted to form a hydrophobic pocket by folding of the four charged amino acids around the hydrophobic phenylalanine residue, a structure that may be involved in direct contact with gp120 (315, 391) (Fig. 3).

The MHC II- and gp120-Binding Sites on CD4 Are Distinct but Overlapping

The ability of gp120 to inhibit interactions between CD4 and class II MHC molecules was first addressed by delineation of the MHC-binding regions of CD4 in comparison with those involved in gp120 interaction. Homolog-scanning mutagenesis on human CD4 has allowed the mapping of both the MHC class II- and gp120-binding sites (71, 253). In a functional assay, mutated CD4 molecules were expressed in a $CD4^-$/$CD8^-$ murine T-cell hybridoma expressing a TCR specific for mouse class I MHC. Activation and interleukin-2 production by this recombinant T-cell hybridoma were dependent on the interaction of both the TCR and CD4 with class I and human class II MHC determinants, respectively, expressed on target cells (253). Other work involved an adhesion assay based on cellular interaction between COS-1 cells transfected with mutated CD4 molecules and a B-cell line expressing human class II MHC determinants (71).

Both groups demonstrated that the binding site for MHC class II on CD4 involved interactions with amino acid residues located in the D1 to D3 domains. In contrast, only amino acids located in the D1 domain were required for gp120 binding (71, 253). Mutated forms of CD4 molecules that could no longer bind to gp120 retained the ability to interact with class II MHC and vice versa, indicating that the gp120- and MHC II-binding sites on CD4 are separable (253, 254). However, mutations in the CDR2 region (D1 domain) equally impaired gp120 and MHC class II binding to CD4, indicating that there is a substantial overlap between the gp120- and MHC II-binding sites on CD4 (43, 314, 355). These contrasting results may be attributable, in part, to the cellular assays used to address the MHC II-binding competence of the CD4 mutants. Mutation of sequences in the D2 and D3 domains that are not directly involved in MHC II binding may affect MHC II binding through conformational changes in CD4 (71). More importantly, the CDR3 region of CD4 plays a role in essential *cis* interactions with molecules involved in T-cell activation (295). Mutations in the D1 domain of CD4 that affect these *cis* interactions, without perturbing MHC II contact, could conceivably generate artifactual results in a functional assay based on T-cell activation. Nevertheless, these studies show that the extent of the MHC II-binding site, which surrounds the D1 and part of the D2 domains, does not allow simultaneous binding of the two molecules on CD4 (130, 190, 316), even though differences may exist among some residues directly involved in gp120 versus class II MHC binding on CD4.

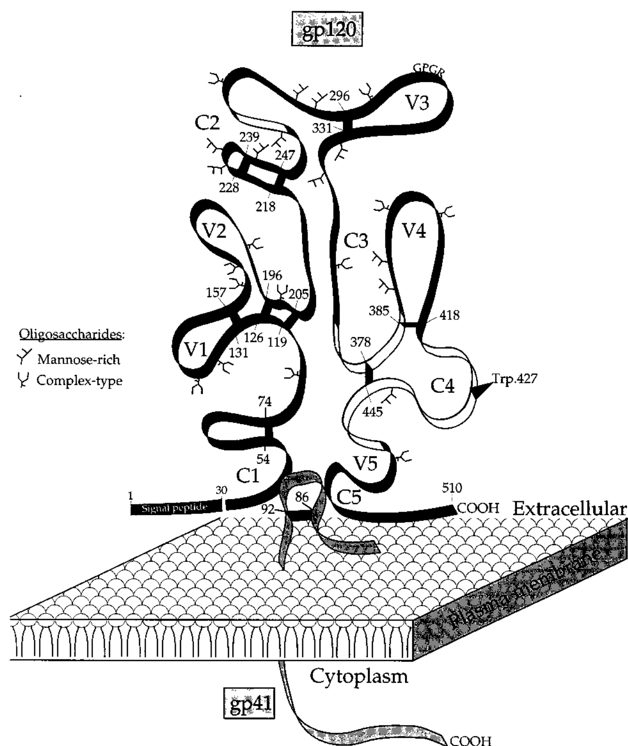

FIG. 4. Schematic representation of gp120 and its noncovalent interactions with gp41. The viral envelope glycoproteins are shown at the surface of infected cells or viral particles. The constant regions, C1 to C5, are separated by more-variable regions termed V1 to V5. These regions are stabilized by intramolecular disulfide bonds represented in black. The positions of the disulfide bonds and the glycosylation sites are from reference 266. The hypervariable domain V3 involved in HIV-1 fusion and proteolytic cleavage during infection is represented along with the hyperconserved sequence GPGR at the tip of the loop. The location of regions in the C2, C3, and C4 domains, shown by mutagenesis and epitope mapping to contain residues important for CD4 binding, are represented by white sections on the ribbon structure. The C1 and C5 domains contain the amino acid determinants involved in interaction with the extracellular cysteine loop of the transmembrane gp41.

Binding Site for CD4 on gp120

The HIV-1 gp120 envelope protein has a complex secondary structure stabilized by disulfide bonds between conserved cysteine residues (Fig. 4). Extensive variability is apparent in five discrete gp120 domains termed V1 to V5, as shown by sequence comparison among various HIV-1 isolates (505). Conserved amino acid sequences, termed C1 to C5, separate these hypervariable regions (Fig. 4).

Several of these domains interact with each other to form the complex secondary structure of gp120 (132, 320, 321, 503, 504). Consequently, probing the CD4-binding site by amino acid deletions and substitutions in gp120 is often impeded by the extensive structural changes caused by the mutations. Although the N-terminal part of gp120 was originally thought to be involved in CD4 binding (332, 453), it is now accepted that the vast majority of residues important for CD4 binding are located in the C-terminal half of gp120, from the C2 to the C4 domains (117, 360, 361).

Neutralizing antibodies that interfere with the binding of CD4 to native gp120 have been extensively used to further map the CD4-binding site. Such antibodies, isolated from AIDS patients or mice immunized with gp120, were tested against different point mutants of gp120 for their ability to neutralize infection. The same gp120 mutants were also tested for their ability to bind soluble CD4 in vitro. Cumulative results from these studies defined three major regions of gp120 important for CD4 binding. They consist of residues 256 to 262 in the C2 domain, residues 368 to 389 in the C3 domain, and residues 421 to 457 in the C4 domain (80, 259, 297–299, 377, 450, 461, 462) (Fig. 4). Additional residues in the C5 domain have been reported to also contribute to the formation or stability of the CD4-binding site (461, 462). These latter domains occupy over 200 residues on the linear structure, indicating that a functional CD4-binding site may be formed only upon proper folding of gp120.

In the absence of X-ray crystallography data, the elucidation of the atomic structure of gp120 has relied mostly on antibody epitope mapping. Such techniques have recently revealed that the C2 and C5 domains of gp120 are poorly exposed at the surface of the molecule (320). It is thus likely that residues within these domains contribute to the structure of the CD4-binding site but are not involved in direct contact with CD4. In contrast, the C4 domain and most of the C3 domain protrude from gp120 and may be of particular importance for interactions with CD4 (320).

Mutagenesis of single amino acids in the C3 and C4 domains profoundly impaired CD4 binding and viral infectivity (79, 336). In particular, both conservative and nonconservative substitutions of a tryptophan residue at position 427 were shown to abrogate CD4 binding (79). Substitution of two aspartic acid residues and a glutamic acid residue (at positions 368, 457, and 370, respectively) for positively charged amino acids impaired CD4 binding, as well, without perturbing the conformation of gp120 (336). The complete loss of CD4 affinity, following single-amino-acid changes, indicates that these residues are closely involved in a structure essential for CD4 interactions. Such a structure may be a hydrophobic pocket, consisting of the central nonpolar Trp-427 surrounded by the negatively charged Asp and Glu amino acids (Fig. 4); this is an arrangement reminiscent of that of the CDR2 domain of CD4 involved in gp120 binding (Fig. 3). The fact that the surrounding hydrophilic residues of CD4 and gp120 are opposite in charge suggests that the CDR2 and C4 domains may directly interact (315). This model is compatible with current predictions of gp120 atomic structure (138).

Postentry Events Involving CD4 and Viral Envelope Glycoproteins

After binding to cell surface CD4, HIV-1 gains entry through direct membrane fusion, a pH-independent event (293, 440). HIV-1 fusogenic activity is located in extracellular N-terminal gp41 sequences that are proximal to its membrane-spanning region (133, 244); this area and that of the fusion peptide of paramyxoviruses have strong homology (158). Substitutions within the gp41 fusion peptide, involving replacement of hydrophobic by charged amino acids, impair syncytium formation (243), by disrupting amphipathic α-helical structures of the fusion peptide that normally permit insertion into target cell membranes (479).

Masking the amphipathic regions of gp41 and restriction of gp41-mediated fusion activity may be the consequence of gp120-gp41 association and may persist until the virus and cell membrane are closely apposed (352). Both the C- and N-terminal 30 residues of gp120, located in the C1 and C5 domains, are involved in these gp41 associations (183, 199) (Fig. 4). These regions are believed to form a molecular pocket that provides an anchor site for the cysteine loop present in the extracellular part of gp41 (272, 411). Interaction of gp120 with cell surface CD4, as well as soluble forms of CD4, consisting

only of the extracellular part of the molecule (178, 319), is thought to trigger conformational changes resulting in its dissociation from gp41, thus exposing the fusogenic domain (reviewed in reference 113). Spontaneous shedding of gp120 also occurs, because gp41 is weakly associated with gp120 at the virion surface (148).

In addition to the fusion activity provided by gp41, the third variable domain (V3) of gp120 was shown to be involved in HIV-1 penetration into target cells and syncytium formation (89, 134, 327). The role of this envelope domain in viral infection is further supported by the highly neutralizing activity of antibodies generated in vivo against the V3 loop (204, 205, 317). Since neutralizing antibodies against the V3 principal neutralizing determinant can inhibit HIV-1 infection without interfering with CD4 binding, this suggests that the V3 loop is involved in a postbinding event (426). Amino acid sequences between the two cysteine residues that stabilize the V3 loop are remarkably variable among different isolates of HIV-1. Such variability has been correlated with disease progression (224, 250, 308), tropism for T cells versus monocytes/macrophages (197, 420, 423, 507, 512), and escape of virus from the activity of neutralizing antibodies (313). Sequence variability in the V3 loop can be used to trace the evolution and epidemiology of HIV-1 by comparing V3 loop sequences of different clinical isolates (reviewed in reference 160).

In the crown of the V3 loop, a strongly conserved "GPGR" sequence contrasts with the high variability of this domain (258) (Fig. 4). Amino acid substitutions in the conserved GPGR sequence generated envelope glycoproteins unable to mediate cell fusion, despite normal processing and CD4 binding (135, 168, 198, 343). Sequence homology between the gp120 GPGR sequence and the active site of tryptic protease inhibitors suggests that the role of conserved residues in the V3 loop may be to promote proteolytic cleavage of gp120 upon binding to CD4. Tryptic protease inhibitors as well as antibodies against tryptase TL2, a cell surface serine protease expressed in human $T4^{+}$ lymphocytes, inhibited gp120-mediated syncytium formation (180, 225).

Experiments performed in vitro with purified gp120 showed that tryptase TL2 specifically binds to gp120 in the V3 region (226) and that cleavage could be demonstrated with both tryptase and a related enzyme, thrombin (72). Cleavage of the V3 loop by thrombin was inhibited by neutralizing antibodies against the principal neutralization determinant. This suggests that one possible mechanism of neutralization of HIV-1 infection involves the masking of the gp120 cleavage site (72). Epitope-mapping studies have shown that upon binding of soluble CD4 on gp120, conformational changes important for fusion occur in the V3 loop (399). Since the C4 domain, involved in CD4 binding, interacts with the V3 as well as the V1/V2 domains in the folded gp120 molecule, it is likely that CD4 binding can indirectly induce changes in the conformation of gp120 (132, 510). Such a rearrangement would expose the thrombin cleavage site that is otherwise masked (207), leading to proteolytic cleavage of gp120 and possibly dissociation from gp41, thus exposing the fusogenic peptide (400).

Domains of CD4 important for postbinding events lie outside the gp120-binding site. Fusion of HIV-1 and the cell membrane depends on a degree of flexibility between the D2 and D3 domains of CD4, such that virus can closely appose to the cell surface (55, 178, 252, 399, 468). gp41-mediated syncytium formation, but not binding of CD4 to gp120, was inhibited by each of two MAbs directed against the D3 domain of CD4 (179, 182), possibly through interference with the versatility of the D2-D3 hinge, thereby preventing apposition of the cell membrane and the gp41 fusion domain. MAbs directed against the D2 domain of CD4 also inhibited syncytium formation by preventing structural alterations of CD4 involved in this process (48).

The importance of the flexible hinge between the D2 and D3 domains of CD4 was also studied with chimeric molecules containing the CD4 D1-D2 domains fused to the hinge, transmembrane, and cytoplasmic domain of human CD8. Cells expressing such CD4-CD8 chimeras showed markedly reduced susceptibility to HIV-1 infection and were unable to form syncytia with gp120-expressing cells (362). Delays in virus infection and syncytium formation by the CD4-CD8 chimera were attributed to an inability to undergo rapid conformational changes triggered by gp120 binding (157). The CDR3 region of CD4 was also suggested to be directly involved in the process of membrane fusion through specific interactions with the V3 loop of gp120. This was based on the ability of CD4-CDR3 and gp120-V3 synthetic peptides and MAb directed against the CDR3 to inhibit syncytium formation without affecting gp120 binding (213, 271, 325, 468). However, extensive mutagenesis of the CDR3 and V3 regions and the isolation of anti-CDR3 MAbs that block CD4-gp120 interactions now provide strong evidence that interactions between the CDR3 and V3 domains are not directly involved in the postbinding fusion process leading to viral penetration (45, 78, 318).

BIOLOGICAL EFFECTS OF CD4-gp120 CELL SURFACE INTERACTIONS

Triggering of T-Cell Activation

HIV-1 replication is greatly enhanced by T-cell activation (282). This could result from an activation signal delivered by CD4-gp120 binding at the cell surface. Alternatively, but not exclusively, activation by antigenic stimulation of latently infected T cells may be necessary to establish productive infection.

The notion that HIV-1 infection activates T cells is difficult to reconcile with the fact that HIV-1 can remain latent in resting T lymphocytes for prolonged periods; indeed, in vitro infection of peripheral blood lymphocytes did not require prior activation and the cells remained quiescent after viral penetration (443, 519). However, such nonactivated T cells were unable to establish productive infection, a finding attributed either to partial reverse transcription (519) or to a block in chromosomal integration of complete provirus (443). Glycolipid-anchored forms of CD4 that lack the cytoplasmic domain, and are thus deficient in Lck association, were transfected into nonlymphoid HeLa cells; nevertheless, these cells can be productively infected by HIV-1 (242). Since glycolipid-anchored CD4 cannot participate in T-cell activation (429), this suggests that infection can occur independently of the ability of CD4 to deliver an activation signal. In both cases, activation of latently infected cells could trigger viral expression, indicating that HIV-1 may not directly activate T cells and may rely instead on the immune system to establish productive infection. Human studies have shown that the majority of HIV-1-infected resting $CD4^{+}$ T cells in asymptomatic patients harbored nonintegrated silent provirus (46). Activation of latently infected T cells led to full chromosomal integration and viral replication.

Interaction of purified gp120 with CD4 at the surface of quiescent T cells was shown to induce activation, as monitored by increases in intracellular levels of inositol phosphate and Ca^{2+} and expression of interleukin-2 receptors (241). However, no such results were obtained with quiescent T cells following exposure to either HIV-1 or purified gp120 (189, 338, 435). Despite the absence of T-cell activation, monomeric and

cross-linked gp120 molecules have been shown to induce dissociation of the CD4-p56^{lck} complex and activation of Lck after binding to the cell surface CD4 (73, 186, 210, 435). These observations indicate that cross-linking of cell surface CD4 by gp120 activates the p56^{lck} protein tyrosine kinase but is unable to promote T-cell activation, probably because of the lack of recruitment of the relevant Lck substrates in the TCR.

Although there is substantial evidence that HIV-1 infection per se does not trigger T-cell activation, the virus may preferentially target a subset of T cells that are easily activated subsequently. Such a subpopulation of memory T cells may be 4 to 10 times more susceptible to HIV-1 infection in vitro than are naive T cells (404). This may be related to the selective depletion of memory T cells in patients (473). Infected memory cells have been shown to contain integrated provirus, suggesting that their state of activation provided the necessary requirements for establishment of productive infection (58).

Direct cellular activation by HIV-1 may play a role in this process. The particular isoform of CD45 expressed by memory cells is always associated with the CD4-Lck complex (98). Therefore, sustained dephosphorylation of the inhibitory Tyr-505 of Lck by CD45 in memory cells might facilitate full catalytic activation of Lck generated by cross-linking of CD4. Since HIV-1 infection can induce multimeric aggregation of CD4 at the cell surface (109), activation of Lck by HIV-1 binding to memory cells might account for their greater susceptibility to infection.

Inhibition of T-Cell Activation by gp120 and Induction of Apoptosis

Selective impairment of CD4$^+$ T lymphocyte function accounts for the profound immunodeficiency characteristic of AIDS (120). However, only a small proportion of circulating T cells actively express HIV-1 in vivo, indicating that the killing of single infected cells is unlikely to account solely for the profound immunodeficiency associated with this disease (161, 405). This has led to the suggestion that HIV-1 might indirectly affect the immunological competence of noninfected CD4$^+$ T cells.

In this context, gp120 and MHC II compete for a distinct but overlapping binding site on CD4 (130, 314, 315). In addition, the CD4-MHC II interaction is considerably weaker than that of CD4 and gp120 (affinity constant, 4×10^{-9} M [259]) (316). It is thus likely that gp120 can displace MHC II interactions with CD4 (Fig. 5). In support of this notion, it has been shown that preincubation of purified gp120 with murine T-cell lines that express human CD4 abrogated adhesion and delivery of an activation signal by MHC class II-expressing target cells (97, 386, 491). Binding of soluble gp120 on the surface of noninfected CD4$^+$ cells can thus render such cells unable to participate in immunological responses against a variety of pathogens, including HIV-1.

Binding of purified gp120 to CD4$^+$ T cells can also deliver intracellular signals that inhibit subsequent T-cell proliferation. Incubation of gp120 with human T-cell clones, specific for diphtheria toxin, abrogated stimulation by both specific antigen and anti-CD3 MAbs (53). Since this inhibition could be correlated with the disappearance of the CD4-Lck complex from the cell surface, it is likely that the gp120-driven internalization of the CD4-Lck complex prevented CD4 from contributing to T-cell activation (463). Cross-linking of CD4-bound gp120 at the T-cell surface with anti-gp120 antibody enhanced both CD4 internalization and the inhibition of T-cell activation by anti-CD3 MAbs (311).

Several mechanisms might be involved in the gp120-induced internalization of CD4. Phorbol myristate acetate (PMA), which is an activator of protein kinase C (PKC), can induce serine phosphorylation of CD4, followed by CD4 internalization (4) and lysosomal degradation (353). Binding of HIV-1 to CD4$^+$ lymphocytes also induces the serine phosphorylation and internalization of CD4 by a mechanism thought to involve PKC activation (127). However, purified gp120 did not have this effect (194), suggesting that cross-linking of CD4 by multimeric gp120 on the virion surface may be necessary to induce CD4 internalization through the serine phosphorylation route (Fig. 5). The finding that gp120–anti-gp120 complexes are present at the surface of CD4$^+$ lymphocytes of AIDS patients suggests that these complexes may play a role in immunological dysfunction related to HIV-1 infection (11).

The ability of gp120 to induce CD4 internalization may involve mechanisms whereby noninfected T cells which have cell surface CD4-gp120 complexes are targeted for destruction by cytotoxic T cells. In this context, CD4 is believed to promote the internalization, processing, and presentation of gp120 epitopes at the cell surface in the context of MHC class II (257). The presence of these gp120-MHC II complexes at the cell surface can trigger killing by MHC II-restricted CD4$^+$ cytotoxic T cells (425). A similar effect was obtained without the need for gp120 internalization and processing, when CD4$^+$ T cells were incubated in the presence of soluble gp120 and anti-gp120 antibodies (491).

Binding of gp120 at the surface of CD4$^+$ cells has also been shown to directly transduce inhibitory signals that lead to T-cell anergy characterized by resistance to subsequent T-cell activation. In normal T cells, cross-linking of CD4 in the absence of the TCR induces a negative proliferation signal for T cells (23, 464, 488). gp120 may deliver a similar signal upon binding to CD4, since incubation of human peripheral blood lymphocytes with either virus lysates, purified gp120, or inactivated virions inhibited phytohemagglutinin-induced proliferation (281). Similarly, synthetic peptides overlapping the CD4-binding site of gp120 as well as soluble gp120–anti-gp120 MAb complexes were shown to inhibit the proliferative signal delivered by anti-CD3 MAbs in a T-cell assay system that was independent of MHC class II interaction (77, 269). No internalization of CD4 was detected after incubation with the synthetic peptides, suggesting that the inhibitory potential of gp120 involved the delivery of an “off” signal to T cells by either active negative signaling or inhibition of CD4-TCR interactions (Fig. 5).

Since T-cell activation leads to enhanced viral replication (326, 481), a result of T-cell anergy could be the establishment of silent infection in the absence of immune recognition. This is an important concept in HIV-1 pathogenesis. The involvement of the p56^{lck} protein tyrosine kinase in this process has been suggested (467). Cells that expressed CD4 mutant molecules, unable to associate with p56^{lck}, were more prone to the establishment of productive infection. In contrast, when CD4 was associated with p56^{lck}, binding of HIV-1 at the cell surface delivered a negative signal that significantly decreased the ability of the virus to replicate (467). The involvement of p56^{lck} activity in both positive and negative signaling in T cells could thus reconcile the apparently contrasting results that gp120 binding on cell surface CD4 activates p56^{lck} without triggering T-cell activation (210, 435).

Cross-linking of the CD4-Lck complex in the absence of TCR was further shown to induce apoptosis (21, 181, 329), a single-cell death mechanism characterized by nuclear collapse and DNA fragmentation (reviewed in reference 75). Recent studies indicate that CD4$^+$ T lymphocytes, isolated from AIDS patients at different stages of disease, undergo apoptosis in

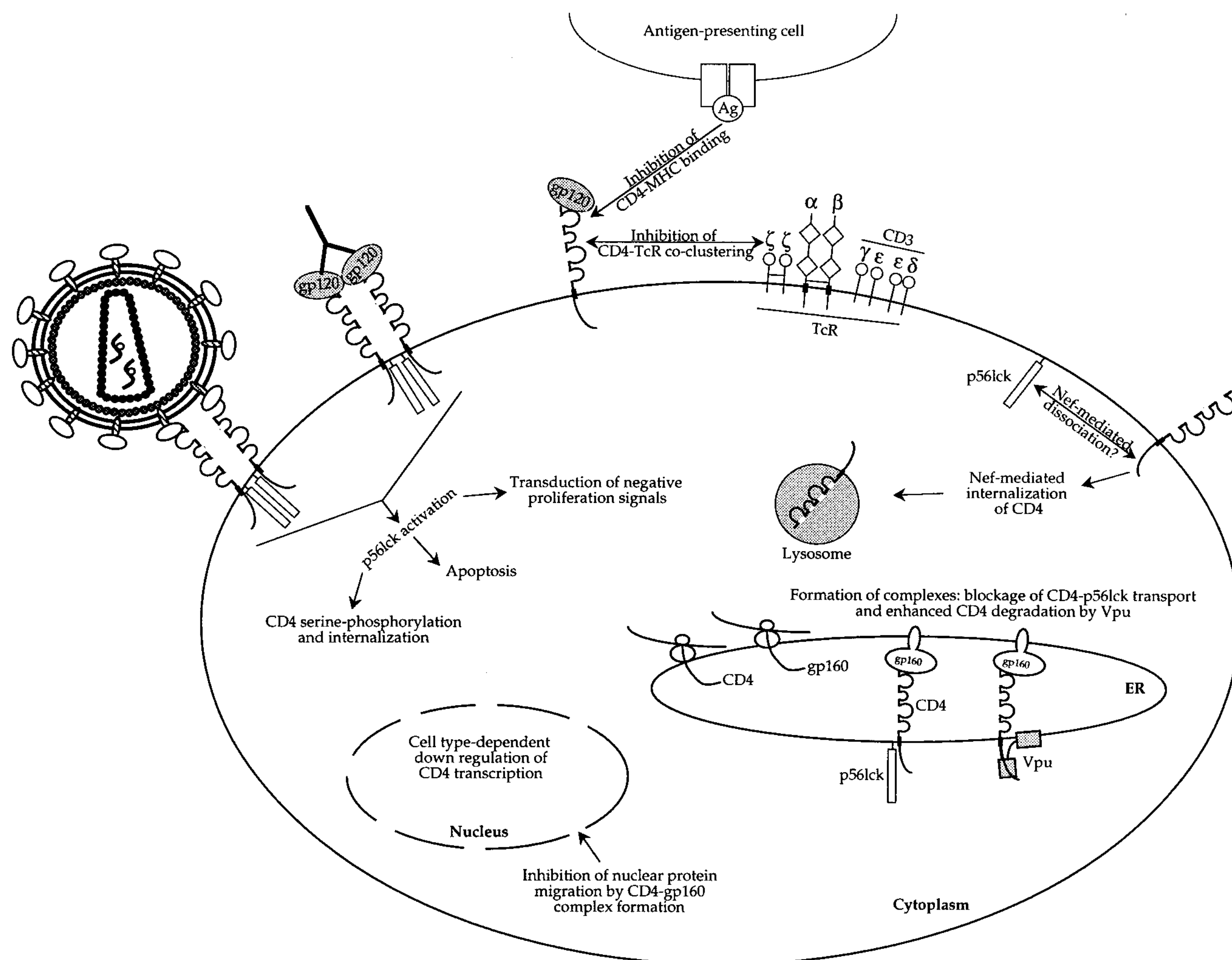

FIG. 5. Extracellular and intracellular interactions between HIV-1 and the CD4 receptor. The potential effects of cell surface cross-linking of the CD4-p56^{lck} complex by gp120 oligomers on the immunological competence of noninfected CD4$^+$ lymphocytes is represented. The ability of monomeric gp120 to block CD4 interactions with the TCR and MHC class II determinants is also shown. In infected cells, several mechanisms account for cell surface depletion of CD4. The three types of major interactions between CD4 and viral proteins are depicted. Complexes between CD4 and the gp160 envelope glycoprotein precursor are trapped in the ER and sensitize CD4 for degradation by Vpu. At the cell surface, the Nef protein induces CD4 internalization and lysosomal degradation.

vitro when stimulated to proliferate through the TCR-signaling pathway (162, 164, 169, 306, 342, 458). Interestingly, similar effects were obtained when human CD4$^+$ T cells, bearing cross-linked gp120 molecules bound to cell surface CD4, were stimulated through the TCR (12, 21, 261) (Fig. 5). This mechanism is similar to that of apoptosis in normal T cells, whereby activation of p56^{lck} by CD4 cross-linking, in the absence of TCR recruitment, can induce cell death (329). The demonstration that gp120-induced apoptosis is dependent on the multimeric structure of this molecule indicates that CD4 cross-linking, as well as p56^{lck} activation, is likely to be involved in HIV-1-induced cell death (412).

Thus, cross-linking of CD4 in the absence of proper recruitment of the TCR (i.e., in the context of MHC II antigen presentation) can induce a period of reversible T-cell anergy (269), during which subsequent activation through the TCR triggers a process of programmed cell death (164). CD4 appears to have a pivotal role in both processes by virtue of its association with the p56^{lck} protein tyrosine kinase. The ability of gp120, either as an oligomer at the viral surface or in the form of antigen-antibody complexes, to artificially cross-link CD4 may amplify the frequency with which noninfected T cells are eliminated.

RETROVIRAL INTERFERENCE

Classification of Retroviruses in Receptor Interference Groups

Identification of viral receptors is a complex process, made difficult by the weak affinity of receptors for viral envelope proteins and low levels of receptor expression. Alternative viral entry routes and the absence of defined function for certain viral receptors add to these difficulties. Thus, most retroviruses are still classified according to patterns of receptor interference (387), i.e., mechanisms whereby productive infection prevents superinfection through a common cellular receptor by exogenous retroviruses of the same envelope type (369, 396, 482, 493). On the basis of intra- or interspecies superinfection interference, each of avian, feline, murine, and human retroviruses have been classified into receptor interference groups (106, 177, 214, 239, 264, 482).

Receptor interference is not limited to retroviral infection; it has been documented for other viruses, including members of the herpesvirus family (51, 59, 208) and even phage lambda (188). Interference patterns and virus pseudotyping have provided key information on the types of receptors used by different retroviruses. In the absence of molecular characterization of the receptor, these assays have provided information on the evolutionary relationships between different classes of retroviruses that use a common receptor (223, 432).

Superinfection interference was first thought to be due to competitive blocking of receptor sites at the cell surface by endogenously produced virus envelope proteins (438). An alternative is that receptor-Env complexes form in the ER during maturation of both proteins. In the latter case, complex formation may lead to impaired maturation and transport of cellular receptors required for infection by viruses of the same envelope type (92, 212, 381). Similar controlling mechanisms may lead to the induction of erythroleukemia by the Friend spleen focus-forming virus (516). Although there is no evidence that the erythropoietin receptor (EpoR) is a cellular receptor for Friend spleen focus-forming virus, intracellular trapping by the gp52 envelope glycoprotein of Friend spleen focus-forming virus may be necessary for virus-induced proliferation of erythroid precursor cells (487).

Retroviral Interference by HIV-1

Research on HIV-1 infection of $CD4^+$ T cells and monocytes/macrophages has led to a more complex picture of retroviral interference. Disappearance of the CD4 receptor from the cell surface usually follows establishment of a productive infection (85, 276, 442). Although formation of intracellular complexes between CD4 and HIV Env proteins impairs CD4 maturation and transport to the cell surface (41, 82, 192, 200), other mechanisms are involved in superinfection interference by HIV-1. Downregulation of CD4 also involves viral proteins distinct from the HIV Env protein, which act at each of transcriptional, translational, and posttranslational levels (143, 192, 394, 501, 502, 518). Removal of cell surface CD4 is apparently necessary and sufficient to block superinfection of cells by both HIV-1 and HIV-2 (264). However, other factors, such as inhibition of de novo reverse transcription, may ultimately be involved as well (122, 455).

Cytopathology Associated with Superinfection

Retroviral interference is a highly conserved process that may be under strict selection pressure, since an inability to block superinfection has been correlated with cytopathology and cell death (103, 382, 457, 494).

Although infection by avian leukosis viruses of subgroups A and E does not functionally alter the host cell, generalized cytopathic effects are associated with subgroups B, D, and F viruses (163). This subgroup-specific cytopathology of avian leukosis virus infection was associated with accumulation of nonintegrated viral DNA (494), resulting from massive superinfection due to reduced capacity to induce receptor interference (495). Inability to protect the host cell from superinfection could be due to the abundance of the cellular receptor, to its lower affinity for viral envelope proteins, or to the existence of secondary receptors that are not downregulated and remain available for superinfection (370).

Complete removal of a receptor that has an essential biological role may prevent superinfection but may also perturb cell metabolism and cause cell death. In the case of MuLV-A, which uses the y^+ amino acid transporter as a cellular receptor, infected cells retain substantial y^+ activity despite the establishment of effective superinfection interference (483, 485). Although MuLV-A infection caused only 50 to 70% downmodulation of cell surface EcoR levels, saturation of the remaining cell surface receptors by extracellular gp70 envelope glycoproteins prevented superinfection. Cell surface receptor bound to gp70 remained functional for amino acid transport across the membrane (483).

Similar mechanisms govern the establishment of noncytopathic infection by HIV-1. Productive infection correlates with cell surface depletion of CD4 after HIV-1 infection (68, 193, 231). By using a model of CEM cells engineered to express reduced amounts of surface CD4, it was shown that the envelope glycoprotein gp160 could induce a state of superinfection resistance essential for the establishment of persistent, noncytopathic infection (442). As with avian leukosis virus, failure to interfere with expression of the receptor led to superinfection that correlated with accumulation of nonintegrated viral DNA and cytopathology (347, 383, 442). Such an event could account for certain brain disorders, e.g., dementia, seen in AIDS patients (345). Although there is a direct correlation between superinfection and nonintegrated viral DNA accumulation (33), it is not clear how extrachromosomal HIV-1 DNA can induce cell killing, although the ability of unintegrated proviruses to synthesize large amounts of viral proteins that accumulate in cells could account for increased cell death (441).

Accumulation of nonintegrated DNA might also be a marker of intense viral replication rather than a direct cause of cytopathicity. In acute infection, a high multiplicity of HIV-1 was shown to induce killing before the appearance of nonintegrated proviral DNA (260). Superinfection by HIV-1 may thus trigger replication of either incoming or resident virus, leading to cytopathicity (228), accompanied by transient accumulation of nonintegrated viral DNA in surviving cells (33).

Downregulation of MHC Antigens as a Means of Immune Evasion

$CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells are both essential components of cell-mediated immune responses against viral infection. The activity of both is dependent on the presence of class I and II MHC at the surface of either infected cells or APC (reviewed in reference 284).

A number of DNA and RNA viruses have evolved mechanisms of escape from immune surveillance. For HIV-1, epitope masking resulting from sequence hypervariability has been shown to be an effective mechanism for immune system evasiveness (509). However, even for the highly variable retroviruses, this mechanism requires multiple rounds of infection to generate viable escape mutants. A more immediate strategy is the direct removal of MHC class I molecules from the surface of infected cells (291). Acute infection of T lymphocytes and monocytic cells by HIV-1 results in the transient downregulation of MHC class I expression (403). Chronically infected monocytic cells showed normal HLA class I surface expression (280), pointing to the transient aspect of this phenomenon. Reduced levels of cell surface MHC I antigens after HIV-1 infection were correlated with lower levels of MHC I mRNA (403), implying that HIV-1 can directly or indirectly affect the activity of the MHC class I gene promoter. This hypothesis is supported by a recent study showing that the two-exon form of Tat can specifically downregulate the activity of the swine class I gene promoter (191). Although the precise mechanism is unknown, similar effects on MHC I transcription have been observed after infection of cells by MoMuLV (155) and adenovirus type 12 (302, 406).

In the latter case, downregulation of MHC I has been shown

to play an essential role in the protection of infected cells from the immune system; this is essential for survival and replication of adenovirus type 12-transformed cells (32, 107). The E1A region of adenovirus type 12 encodes proteins responsible for the specific shutdown of the class I HLA promoter (471, 474) as a result of a decreased rate of initiation (3, 136, 301). Detailed studies of the MHC class I promoter showed that E1A induced the expression of a nuclear repressor which decreased the activity of the promoter by interacting with the class I HLA enhancer element (147, 215, 248). It is not known whether Tat interacts directly with the MHC class I promoter or, as in the case of E1A, induces specific repressors of that promoter. It is also conceivable that posttranscriptional mechanisms, e.g., decreased stability of MHC I mRNA or impaired splicing and transport, are involved in both the Tat- and E1A-mediated downregulation of MHC class I expression (419, 472).

A posttranscriptional level of HLA class I antigen downregulation has also been proposed for HIV-1 (221). The cell surface reduction of MHC I expression in HIV-1-infected cells generated a marked decrease in the capacity of these cells to function as allogeneic targets of $CD8^+$ cytotoxic cells (221). A number of posttranslational mechanisms could account for the reduction in cell surface expression of MHC I after HIV-1 infection. HIV-1 could affect MHC I transport by impairing association with antigenic peptides in the ER (220). Alternatively, mechanisms similar to those used by adenovirus type 2 (19) and both mouse and human cytomegalovirus (25, 91) to downregulate MHC I may be effective.

INTRACELLULAR INTERACTIONS BETWEEN CD4 AND HIV-1

Elimination of CD4 from the surface of HIV-infected peripheral blood lymphocytes was first shown by measuring a decrease in specific immunofluorescence with MAb directed against CD4 (232). Steric hindrance by gp120 could not have been responsible for the result, since the MAb used, i.e., OKT4, recognizes an epitope distinct from the gp120-binding site. Disappearance of the OKT4 epitope correlated with levels of virus replication but was independent of viral cytopathic effect (193, 232). Cell surface CD4 is also expressed by various human monocytoid cell lines, such as U-937 and HL60, that can easily be infected with HIV-1 (68). Chronic infection of these cells also leads to disappearance of cell surface CD4 (68), suggesting that such downmodulation is cell lineage independent. The intracellular mechanisms involved in CD4 cell surface depletion in HIV-infected cells are schematically depicted in Fig. 5.

HIV-1 Infection Downregulates CD4 Transcription

Diminished steady-state levels of CD4 mRNA were demonstrated in a number of chronically infected T-cell lines (149, 192). The degree to which this occurred depended on the extent of virus-induced cytopathology that preceded the establishment of chronic infection. Thus, selective killing of high-CD4-expressing cells could have accounted for this outcome in some cases. Other studies showed, however, that decreased levels of CD4 mRNA can be detected in acutely infected lymphocytes prior to extensive cytopathology (394).

The cellular environment also appears to have a critical role in the efficiency with which HIV-1 regulates levels of CD4 mRNA. In monocytoid cell lines, chronic HIV infection had no effect on steady-state levels of CD4 mRNA, in spite of the disappearance of the CD4 antigen from the cell surface (149). HIV-1 infection may mimic a physiological pathway used by the cell to downregulate CD4 mRNA expression. Agents such as PMA are known activators of intracellular PKC, and they have been used to induce myeloid and lymphoid cell differentiation (52, 81, 94, 95, 170). Treatment of human T lymphocytes or monocytes with PMA results in the rapid phosphorylation of cell surface CD4 followed by internalization and lysosomal degradation of the molecule (128, 194, 353). Similar results were obtained after antigenic stimulation of T cells, indicating that PMA-induced CD4 phosphorylation and downregulation mimicked normal cellular pathways (4, 38). In addition to the posttranslational effect of PMA on CD4 levels, PMA may specifically cause downregulation of CD4 transcription (328, 356, 375). Activation of T cells by binding of gp120 to cell surface CD4 may involve similar pathways to those described for PMA, leading to potentiation of PKC and downregulation of CD4 transcription. Further studies of human and mouse CD4 promoters, as well as the mechanisms involved in downregulation of CD4 transcription by other viruses, will help clarify the effect of HIV-1 infection on CD4 mRNA levels (393, 401, 522).

Formation of Intracellular Complexes between CD4 and gp160

CD4-gp160 intracellular complexes represent an important mechanism, leading to induction of superinfection interference (192, 442). The extent of CD4 surface depletion caused by the formation of such complexes directly correlates with levels of viral activation and production of gp160 (49, 218, 416). Similar mechanisms are responsible for superinfection interference mediated by the Env protein in a number of different animal retrovirus systems (92, 381, 516).

CD4-gp160 complex formation is both necessary and sufficient to impair CD4 cell surface expression in a concentration-dependent fashion (49, 218). HeLa cells transfected with both CD4 and an excess of gp160 expression plasmid contained complexes of CD4 and gp160, but not gp120, impairing the oligosaccharide processing that renders CD4 partially resistant to endoglycosidase H (82). The lack of oligosaccharide processing was shown, by indirect immunofluorescence of doubly transfected cells, to be due to a blockage of gp160-complexed CD4 in the ER (82, 200). Tripartite complexes of CD4, gp120, and gp41 also blocked CD4 in the ER of HeLa cells that transiently expressed CD4 and gp160 (200). However, CD4-gp160 complexes were shown to have greater physiological relevance, since CD4 was also trapped in the ER of cells that expressed cleavage-deficient molecules of gp160 (41).

Association between CD4 and gp160 might render the former susceptible to lysosomal degradation, the fate as well of most of the gp160 that is synthesized in infected cells (498). However, the stability of CD4 is increased in chronically infected U-937 cells, following association with gp160 (41). Hence, blockage and accumulation of CD4 in the ER, and not lysosomal degradation, is likely to explain the absence of CD4-gp160 complexes at the cell surface. CD4-gp160 complexes also block gp160 maturation, leading to progressive accumulation of this protein and impairment of both oligosaccharide trimming and proteolytic cleavage (41). Lysosomal degradation of gp160 is thus not caused by intracellular association with CD4 but, rather, represents the normal outcome of the majority of gp160 molecules. The formation of CD4-gp160 complexes probably results in the blocking of both proteins in the ER, rather than induction of their degradation, since formation of intracellular complexes with CD4 suppresses the high rate of gp160 degradation in lysosomes (41). Findings in

accord with this notion were recently published (501), but they are in contrast with the idea that gp160 molecules, intracellularly complexed with CD4, are proteolytically cleaved to gp120 and gp41 (364).

CD4-gp160 complexes may be deleterious for T cells that express the $p56^{lck}$ protein tyrosine kinase. This T-cell-specific enzyme is associated with the CD4 cytoplasmic tail; when both proteins are present in the ER (83), this occurs within 15 min of synthesis of these proteins, i.e., before formation of CD4-gp160 complexes that are detected 30 min after synthesis of CD4 (41, 82). The generation of ternary complexes between CD4-$p56^{lck}$ and gp160 caused both CD4 and $p56^{lck}$ to be blocked in the ER (83). In the absence of CD4, $p56^{lck}$ was unable to localize at the cell membrane; this suggests a dual role of gp160 complexes on T-cell signaling machinery (83).

HIV-1 relies on the cellular biosynthetic machinery for viral replication and production of progeny. It is thus conceivable that the cytopathicity caused by accumulation of large amounts of intracellular CD4-gp160 complexes may have deleterious consequences for viral replication as well. Formation of such complexes may therefore not be an ideal mechanism whereby HIV-1 downregulates the expression of CD4; rather, such complex formation may be an obligatory side effect of the high affinity between CD4 and gp160. In this regard, U-937 cells transfected with env manifested cytotoxicity in proportion to levels of gp160 synthesis and formation of intracellular complexes (237). The localization of CD4-gp160 complexes around nuclear pores was shown to interfere with transport of large proteins bearing a nuclear targeting signal, a process that correlated with some cytopathic effects of HIV-1 infection (235, 236).

Regulation of CD4-gp160 Complexes and CD4 Degradation by Vpu

The viral protein U (Vpu) ORF encodes a well-characterized HIV-1 protein (74, 289, 447). Located between the first exon of *tat* and the *env* gene in the viral genome, Vpu is translated from a bicistronic mRNA that also contains the downstream *env* (414). Translation of gp160 is thus dependent on the leaky scanning of the upstream *vpu* AUG.

A number of infectious clones derived from the HIVIIIb laboratory strain of HIV-1 lack Vpu expression (74). In most cases, the *vpu* ORF is rendered inactive by mutation of the AUG initiation codon. The selection of revertant viruses, in which the initiation codon is restored, might represent a form of Vpu regulation. In contrast, the *vpu* ORF is absent in the closely related viruses HIV-2 and SIV (74), with the exception of the SIV_{cpz} isolate whose genomic organization is similar to that of HIV-1, including the presence of Vpu-like sequences (195).

Although the *vpu* ORF is predicted to encode a protein of 81 amino acids, immunoprecipitation studies showed that Vpu migrates as a 15- to 16-kDa protein on reducing SDS-polyacrylamide gels (74, 447). A protein with a similar molecular mass is recognized by sera of HIV-1-infected patients, indicating that Vpu is produced during the course of HIV-1 infection (74, 446). Discrepancies between the predicted size and apparent molecular mass of Vpu are not attributable to additional Vpu-encoding exons, as in the case of Tat or Rev (447). Indeed, when Tricine-containing SDS-polyacrylamide gels were used, the apparent molecular mass of Vpu was identical to the calculated molecular mass of 9 kDa (409), suggesting that the anomalous gel mobility of this protein is due to the Laemmli buffer system. Translation of Vpu in an in vitro system in the presence of microsomal membranes showed that Vpu is cotranslationally integrated into membranes. However, the relative mobility of Vpu was unaffected by the presence of microsomes, indicating that glycosylation had not occurred (446). $^{32}P_i$ metabolic labeling of cells, transfected with the Vpu-positive pNL43 infectious clone, showed that a phosphorylated protein corresponding to Vpu could be immunoprecipitated with both an HIV-positive serum and a monospecific Vpu antiserum (446). Accordingly, two serine residues at positions 52 and 56 in the cytoplasmic domain of Vpu were shown to be phosphorylated by the ubiquitous casein kinase II (409).

Infection of A3.01 T cells with pNL43 or the pNL43/U_{35} Vpu^- infectious clone showed that absence of Vpu did not affect the kinetics of HIV-1 infection but reduced progeny levels by about sixfold (447). Similar results were obtained with Jurkat cells, following infection by isogenic BH10 infectious clones, which differed only in terms of presence of the *vpu* initiation codon (459). Enhanced particle release with Vpu was due to increased secretion of viral proteins in the culture medium. Pulse-chase analysis of proteins produced in A3.01 cells infected by pNL43 or the Vpu^- pNL43/U_{35} viruses showed no role of Vpu in the kinetics of Gag and Env processing (446). However, cells lacking Vpu showed a 20% decrease in relative abundance of secreted p24 capsid protein over a single round of viral replication (446). The presence of Vpu affected neither the intracellular pool of viral protein nor the rate of gp120 secretion (446), indicating that the mechanism of Vpu action may involve increasing the local concentration of Gag proteins at the plasma membrane. Cell fractionation and electron microscopy showed that in the absence of Vpu, a large proportion of cell-associated reverse transcriptase activity was contained in virion structures still attached to the plasma membrane (i.e., blocked in a late stage of maturation) (233). Intracellular budding of aberrantly formed viruses, a pattern that was previously shown to be characteristic of macrophage infection (337), was also observed. Both accumulation of intracellular virions and retention of particles at the plasma membrane may account for the reduced levels of particle release in the absence of Vpu. The mechanism by which Vpu increases particle release is independent of the expression of either CD4 or gp160 (150, 513).

Vpu Induces Degradation of ER-Resident CD4

Since Vpu and gp160 are translated from the same bicistronic mRNA species, it is tempting to speculate that these two proteins may have a functional relationship. Proteolytic processing of gp160 was enhanced by coexpression of Vpu and CD4 in HeLa cells (501). However, Vpu did not act directly on gp160 but, rather, induced the disruption of CD4-gp160 complexes, causing gp160 to be released into its normal maturation pathway (501). Disruption of CD4-gp160 complexes by Vpu was attributable to intracellular degradation of CD4, whose half-life was reduced from 6 h to 12 min (502). CD4 molecules in the ER that were associated with gp160 were preferentially degraded in the aftermath of Vpu expression, but the requirement for complex formation was overcome by the use of brefeldin A, which blocked CD4 in the ER. Therefore, the contribution of gp160 to this process apparently involves trapping CD4 in the ER, in which Vpu-mediated degradation takes place (502).

To define the mechanisms involved, CD4 and Vpu were cotranslated in microsomal membranes in the absence of other viral components (61). This in vitro system reproduced previous findings on Vpu-mediated CD4 degradation obtained in HeLa cells, although the rate of degradation was reduced in vitro. Degradation of CD4 by Vpu required both proteins to be

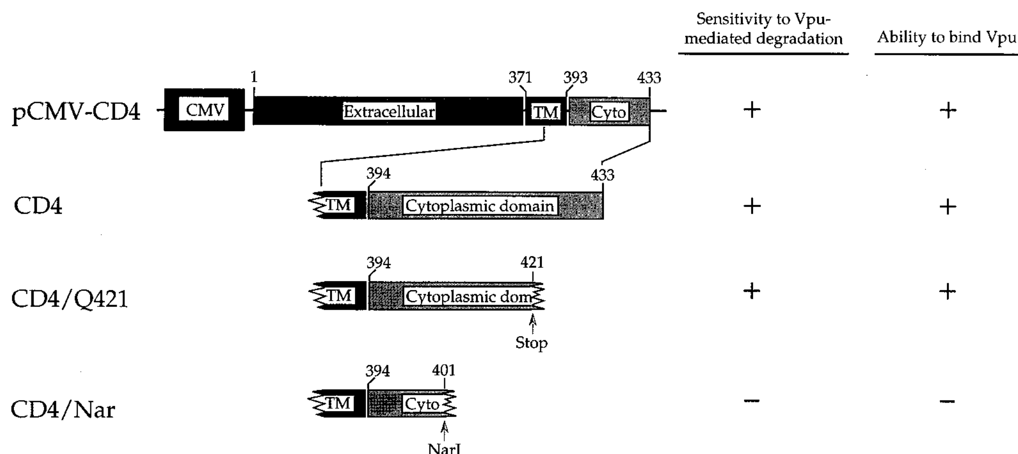

FIG. 6. Amino acid sequences between residues 401 and 421 in the cytoplasmic domain of CD4 are required for binding to Vpu and degradation. The stability of CD4 mutants, lacking the 32 C-terminal residues in the cytoplasmic domain, is not affected by the presence of Vpu. This correlates with impaired ability to physically interact with Vpu, indicating that binding of Vpu to the CD4 cytoplasmic domain triggers processes leading to CD4 degradation.

inserted into the same membrane compartment and was independent of CD4 glycosylation status (61), suggesting that specific target sequences might be present in CD4. The fact that the C-terminal hydrophilic portion of Vpu is located on the cytoplasmic side of membranes (279, 446) suggested that the cytoplasmic tail of CD4 might contain target sequences for Vpu-mediated degradation (61). This was confirmed by using chimeric molecules in which the cytoplasmic tail of CD8, not sensitive to Vpu-mediated degradation (265), was replaced by the corresponding domain of CD4 (499). In this context, the presence of the CD4 cytoplasmic tail was shown to be necessary and sufficient to trigger degradation by Vpu.

To further define the amino acid sequences in the CD4 cytoplasmic tail required for degradation by Vpu, the stability of CD4 mutants with truncated cytoplasmic tails was assessed (Fig. 6). Deletion of the C-terminal 13 amino acids of CD4 had no effect on Vpu-mediated degradation. However, the failure of Vpu to affect stability of a mutant CD4 bearing a 32-amino-acid truncation of the cytoplasmic tail suggested that sequences located between amino acids 402 and 420 of CD4 were important in this regard (61). In HeLa cells, soluble CD4 as well as soluble CD4 molecules anchored to membranes through heterologous transmembrane regions were protected from Vpu-mediated degradation (265, 499). The use of a panel of CD4 cytoplasmic tail deletion mutants confirmed the existence of a discrete target sequence that confers sensitivity to Vpu-mediated degradation. This region encompasses amino acids 418 to 425 and partially overlaps a previously reported functional domain (61, 265) (Fig. 8).

Chimeric molecules, in which the transmembrane and cytoplasmic domains of gp160 were replaced by corresponding regions of CD4, were also used to define the putative Vpu target site (480). A particular sequence (414-LSEKKT-419) was defined as the minimal element in the CD4 cytoplasmic tail needed for efficient Vpu-mediated degradation of the chimera (480). However, whether transfer of this minimal sequence is sufficient to confer Vpu sensitivity to heterologous proteins is unknown. Recent reports have shown, in the context of chimeras of CD4 and gp160, that the transmembrane domain of CD4 can participate in the susceptibility of the latter to Vpu-mediated degradation (365).

The molecular mechanisms involved in Vpu-mediated degradation of CD4 remain obscure. However, the presence of specific target sequences in CD4 suggest that Vpu might trigger degradation by direct interactions with the former molecule. Indeed, coimmunoprecipitation experiments showed that Vpu binds specifically to the cytoplasmic tail of CD4 in a region overlapping amino acid residues 402 to 420, previously shown to be important for degradation (42) (Fig. 6). Since CD8-CD4 chimeric molecules containing only the transmembrane domain of CD4 were unable to bind Vpu, it is likely that the two molecules interact through their respective C-terminal cytoplasmic domains.

The hydrophilic domain of Vpu has been predicted to form an α-helix–turn–α-helix structure with the phosphorylation sites located between the two amphipathic helices (408) (Fig. 7). This theoretical prediction was confirmed by studying the solution structure of synthetic Vpu fragments by a combination of circular dichroism and ^{1}H nuclear magnetic resonance spectroscopy (184). It is thus possible that CD4-Vpu complexes are stabilized through interactions between the amphipathic α-helical domains of Vpu and the cytoplasmic domain of CD4, for which an α-helical secondary structure has also been predicted (129). Although CD4-Vpu binding appears to be essential to trigger degradation (42), the subsequent steps leading to CD4 proteolysis remain to be defined.

By way of analogy to the degradation by the human papillomavirus type 16 E6 protein of the p53 tumor suppressor (402), the ubiquitin pathway of protein degradation might be involved (reviewed in reference 206). The latter is highly selective and mediates the elimination of abnormal or short-lived cellular proteins. Proteins are sensitized for degradation by covalent attachment of multiple units of the 76-residue ubiquitin polypeptide at lysine residues, a process that requires ATP. Polyubiquitinated proteins are subsequently degraded by a multicatalytic protease complex (the proteasome) that also requires ATP for its function (156). The ATP requirement is a hallmark of the ubiquitin pathway; most proteases do not require ATP with the hydrolysis of peptide bonds being thermodynamically favored. Vpu-mediated degradation of CD4, similar to that of p53 degradation by the E6 protein (54), was ATP dependent in a cell-free system (61). Other similarities between CD4 and p53 degradation may also indicate some resemblance between the two mechanisms. Vpu forms specific complexes with its target protein, is unaffected by the degradation process (61), requires ATP for degradation (60), and,

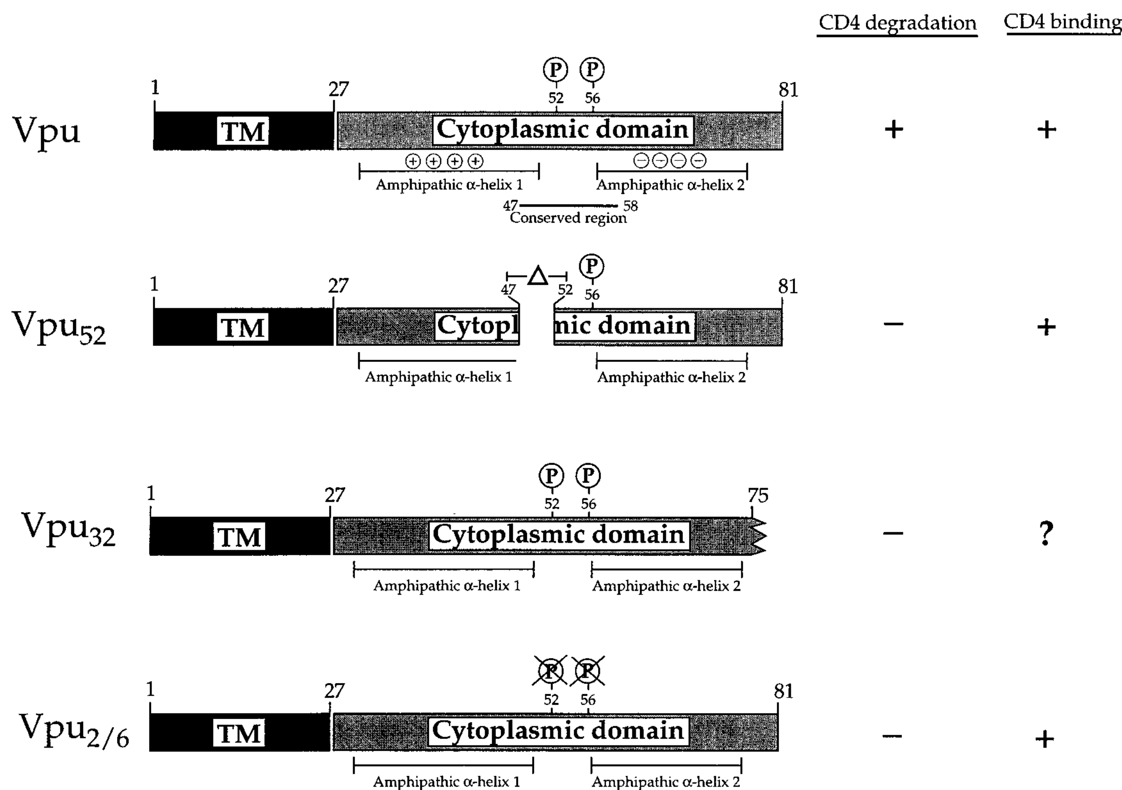

FIG. 7. Distinct Vpu domains are involved in binding and degradation of CD4. Deletions in the conserved region of Vpu (Vpu_{52}) or in the C-terminal portion (Vpu_{32}) generate mutant proteins unable to degrade CD4. Similarly, the removal of the two conserved serine phosphorylation sites at positions 52 and 56 affects CD4 degradation. The ability of all the mutants tested to interact with CD4 indicates that the CD4-binding region in Vpu is distinct from the catalytic region and is likely to lie within the α-helical structures. Vpu mutants depicted in this figure have been described in detail previously (61, 410).

similarly to p53 degradation, functions both in whole-cell systems and reticulocyte lysates (61, 402).

There is increasing evidence that while the degradative function of Vpu specifically targets CD4, the Vpu-mediated increase of viral release involves less-specific mechanisms that can also influence the liberation of Gag proteins from viruses that naturally lack a Vpu activity (159). Evidence that distinct biological activities of Vpu are involved in CD4 degradation and particle release has also come through use of mutant Vpu proteins that lack phosphoacceptor sites at positions 52 and 56 (410). While nonphosphorylated Vpu was no longer able to degrade CD4, the positive effect on viral release was only partially affected by the mutation (Fig. 7). In contrast, retention of wild-type Vpu in the ER with brefeldin A completely blocked its activity on particle release (410) but enhanced CD4 degradation (502). Vpu thus appears to have two distinct biological functions that differ in their specificity and mechanism of action and take place in different cellular compartments.

Examination of the structural features of Vpu may also contribute to clarification of its biological function. Vpu is structurally related to the influenza virus M_2 protein (233, 447), a phosphoprotein that forms homotetrameric structures that function as a proton channel (105, 187, 358, 449). Vpu is also a phosphoprotein that forms higher-order structures consisting of at least four noncovalently linked molecules (279). However, M_2, unlike Vpu oligomers, is stabilized by disulfide bonds (279, 449). Moreover, M_2 is expressed at the plasma membrane and is packaged into virions, whereas Vpu is located mainly in the perinuclear region (233, 521) and is not virion associated (445).

Receptor Interference by the Nef Protein

The *nef* gene, which is specific to primate lentiviruses, overlaps the 3′ end of the *env* gene and approximately half of the 3′ long terminal repeat and is transcribed as a 2-kb doubly spliced mRNA (10). Translational and posttranslational modification of the HIV-1 Nef produces a 27-kDa phosphoprotein that associates with membranes through an N-terminal membrane-associated myristylation (10, 131, 172). *nef* mRNA is abundantly expressed early after HIV-1 infection as well as in latently infected cells of different lineages (234, 378, 466), raising the possibility that Nef plays a role in the establishment of chronic infection by HIV-1 (6, 62, 174, 230, 331). Nef may be able to act on specific transcription factors, such as NF-κB and AP-1, known to be essential for efficient activation of the HIV-1 long terminal repeat (326, 481). The finding that Nef inhibits the induction of NF-κB following T-cell activation may indicate that the former acts by preventing the activation of HIV transcription rather than as an authentic transcriptional repressor (330). Lack of NF-κB induction, following activation of Nef-expressing cells, may be caused by a specific inhibition by Nef of activation signals transmitted through the TCR complex (22, 274). Such an effect could result from the uncoupling by Nef of the TCR and cofactors critical for T-cell activation.

The ability of Nef to physically interact with each of CD4, $p56^{lck}$, and the mitogen-activated protein kinase (300) indicates that Nef may inhibit mitogenic signals through its ability to sequester proteins involved in T-cell activation. Nef can positively influence the induction of viral replication following mitogenic stimulation of infected quiescent peripheral blood

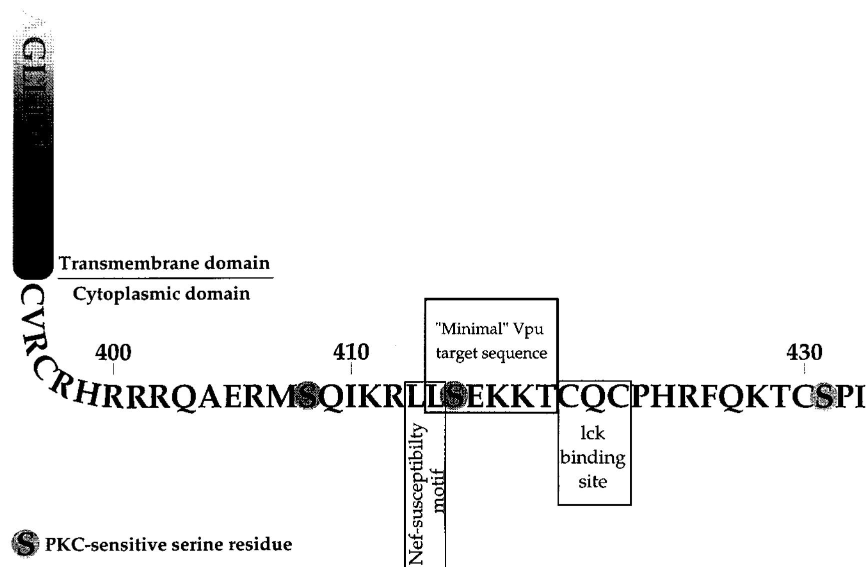

FIG. 8. Residues in the cytoplasmic tail involved in the regulation of CD4 stability and cell surface expression. Amino acid residues in the CD4 cytoplasmic tail important for Nef-, $p56^{lck}$-, and PKC-mediated cell surface downregulation are distinct but remarkably clustered. The minimal sequence required for Vpu-mediated degradation of CD4 is also located in the same region. It thus appears that Vpu, Nef, and $p56^{lck}$ all compete for overlapping binding sites on CD4.

lymphocytes (65, 93, 310, 436, 460, 520). When located at the plasma membrane, chimeric molecules between CD8 and Nef enhanced T-cell activation through the TCR; expression of the chimera in the cytoplasm had the opposite effect (28). The importance of subcellular localization for the function of Nef might clarify the biological relevance of the 25-kDa form of the protein that lacks the N-terminal myristylation domain and is thus unable to associate with membranes (523).

Efficient replication of the related SIV in rhesus monkeys is highly dependent on the presence of an intact *nef* gene (222). Animals infected with Nef^+ SIV showed efficient viral replication and developed typical AIDS pathogenesis. In contrast, monkeys inoculated with Nef-deleted viruses developed antibody responses but produced no detectable circulating virus. The importance of the *nef* gene in disease progression was further shown by the systematic reversion of mutations that introduced translation termination codons in the *nef* ORF of SIV (222). Similar conclusions were reached when HIV-1 and a model of mice with severe combined immunodeficiency (SCID mice) implanted with human fetal thymus were used (203). Moderate viral replication, mediated in part by Nef, could allow the virus to colonize before a high rate of viral protein expression triggers vigorous immune responses (330).

The effect of Nef on expression of cell surface CD4 is now well established. Both HIV-1 and SIV have been shown, both in vitro and in transgenic mice, to induce an early state of superinfection immunity through a Nef-mediated downregulation of cell surface CD4 (30, 44, 172) (Fig. 4). This effect is independent of changes in the transcription and translation of CD4 and does not involve the serine phosphorylation of CD4, showing the noninvolvement of PKC activity (141, 143). Several alleles of Nef, derived from either HIV-1 molecular clones or clinical isolates, were shown to efficiently downregulate CD4 cell surface expression (17, 283, 413).

However, the Nef domains important for CD4 downregulation have not been fully defined. N-terminal myristylation, and thus membrane association, is essential (283), as are the first 20 amino acids of Nef, since the 25-kDa form of Nef is defective for CD4 downregulation (167). In contrast to gp160 and Vpu, which operate in the ER, Nef does not affect the normal maturation and transport of CD4 to the cell surface (395). Rather, Nef acts at the cell surface to mediate the internalization and lysosomal degradation of CD4 (7, 18, 395).

Further studies with CD8-CD4 chimeras showed that the cytoplasmic domain of CD4 is sufficient for Nef-mediated internalization of CD4 (7, 18). These studies also defined a small amino acid sequence in the CD4 cytoplasmic tail, including the dileucine motif at positions 413 and 414, that is required for CD4 internalization by Nef (7, 18). A similar motif is also present in the cytoplasmic tail of the CD3 γ and δ chains and the IgG Fc receptor and is involved in the internalization and lysosomal degradation of these proteins (100, 196, 267). This dileucine motif is also conserved in mouse CD4 (L3T4) (278), suggesting that Nef may also downregulate L3T4 (17).

The major mechanism responsible for phorbol ester-induced CD4 endocytosis in lymphoid cells involves PKC-phosphorylation of serine residues at positions 408 and 415 (421, 422) and correlates with dissociation from the $p56^{lck}$ protein tyrosine kinase (351, 430). In nonlymphoid cells, CD4 is constitutively internalized by entering coated pits at the plasma membrane (348). The finding that in lymphoid cells, the association with $p56^{lck}$ excludes CD4 from the coated pits introduces the notion that the protein kinase regulates the rate of CD4 internalization (349, 350). Since Nef-mediated endocytosis and degradation of CD4 have little effect on steady-state levels of $p56^{lck}$ (18), it is conceivable that CD4 endocytosis results from a Nef-mediated dissociation of $p56^{lck}$. Since CD4 endocytosis by Nef is phosphorylation independent (143), dissociation of $p56^{lck}$ would have to involve direct competition by Nef for a binding site on the CD4 cytoplasmic tail. Although the proximity of the Nef-responsive dileucine motif and the $p56^{lck}$-binding site suggests that binding of these two molecules is mutually exclusive (Fig. 8), this has not been validated by the isolation of complexes between CD4 and Nef (7, 18). Therefore, it cannot be excluded that Nef uses alternative pathways for CD4 endocytosis that do not require prior serine phosphorylation of CD4 or dissociation of $p56^{lck}$ (421). In this regard, ganglioside-induced internalization of CD4 does not require

serine phosphorylation, and although $p56^{lck}$ dissociation is observed, it has been shown to be the consequence and not the cause of CD4 endocytosis (372).

Several biological roles for Nef-mediated endocytosis of CD4 can be envisioned. In contrast to gp160 and Vpu, which operate in the ER to prevent cell surface expression of CD4, Nef mediates the endocytosis of CD4 molecules that normally transit the Golgi network to reach the plasma membrane. Nef might act as a "tightening" factor that ensures complete cell surface depletion of CD4 molecules that escaped both gp160 and Vpu blocks. Unlike gp160 and Vpu, Nef is expressed at high levels early in the viral life cycle (84) and, as shown for SIV, is able to yield superinfection immunity (30). The early removal of cell surface CD4 might prevent the onset of high viral replication and cell death associated with superinfection. Correlation in Nef-expressing transgenic mice between reduced cell surface expression of CD4 and altered T-cell activation (427) suggests that Nef may contribute to silent infection by preventing HIV-1 long terminal repeat activation (407, 465). Finally, the ability of Nef to diminish the cell surface expression of mature envelope protein (413) could contribute to the masking of infected cells from the immune system.

CONCLUSION

The initial stage of HIV-1 infection of target cells involves high-affinity interactions between the viral envelope glycoprotein gp120 and the CD4 cellular receptor. However, interactions between HIV-1 and CD4 are not limited to this initial step. Both intracellular and cell surface interactions between HIV-1 and CD4 persist during the course of infection, resulting in perturbation of the central function of CD4 as a coreceptor for T-cell activation. The gp120 envelope glycoprotein, as part of the viral particle, in complexes with anti-gp120 antibodies, and in soluble form, can interact with CD4 molecules at the surface of noninfected cells. The presence of gp120 at the cell surface can render CD4 lymphocytes susceptible to immune cytolysis. Second, activation of $p56^{lck}$ activity, by cross-linking of cell surface CD4 by multimeric gp120, can induce both T-cell anergy and apoptosis. Such "*trans*" effects of HIV-1 on the competence of the immune system are thought to be involved in HIV persistence and immune escape.

A second major level of HIV-1–CD4 interactions occurs intracellularly in infected cells, through establishment of a state of superinfection immunity following downmodulation of the CD4 receptor at the cell surface. At least three gene products of HIV-1 are involved in this process. Both gp160 and Vpu act in the ER and may synergize to trap and degrade CD4 before it reaches the plasma membrane. Nef acts on cell surface CD4 to induce its internalization and lysosomal degradation. Interestingly, synthesis of both gp160 and Vpu is dependent on prior expression of the *rev* gene; therefore, both participate in CD4 downmodulation during the late structural phase of HIV-1 replication. In contrast, the participation of *nef* in this process occurs in *rev*-independent fashion during the early phase of HIV-1 replication, suggesting that this constitutes an active form of superinfection interference.

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