HUMAN IMMUNODEFICIENCY VIRUS TYPE 1-ASSOCIATED CD4 DOWNMODULATION

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I. INTRODUCTION

The first step in viral infection is the attachment of viral surface proteins to the cell membrane through specific cellular receptors. One or several viral proteins may be required to mediate this attachment step. Both enveloped and nonenveloped viruses are surrounded by multiple copies of surface proteins, which permit multivalent interactions with cellular receptors to occur.

Receptor specificity is a major determinant of cell and tissue tropism and determines the host range of viruses. The various constituents of

cell membranes that can serve as viral receptors include carbohydrates, lipids, and proteins. Among nonprotein components, the best known may be the sialic acid receptors for the influenza virus hemagglutinin that are present on host cell glycoproteins and glycolipids (Weis *et al.*, 1988).

The following membrane proteins are known to serve as viral receptors: several members of the immunoglobulin superfamily [CD4, intercellular adhesion molecule type 1 (ICAM-1), carcinoembryonic antigens, and major histocompatibility complex (MHC) I antigens], two permeases, an integrin [VLA-2 (very late activation-2)], a metalloprotease (aminopeptidase N), a cellular adhesion molecule (laminin receptor), and a receptor for complement fragments C3dg and iC3b (CR2) (Table I).

The viral receptors listed in Table I have been shown to possess biological relevance by the following important criterion: namely, transfection of DNA that encodes receptor activity, into cells that do not express relevant receptors and that are ordinarily nonpermissive for infection, conferred susceptibility. Two other important criteria of biological relevance are the blocking of viral infectivity by antireceptor antibodies and soluble receptor derivatives. On the basis of these criteria, three retroviral receptors have been identified and include the well-characterized CD4 molecule, for human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2) and simian immunodeficiency viruses (SIVs) (Clapham *et al.*, 1989; Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1986; Sattentau *et al.*, 1988); a basic amino acid transporter termed ecoR or Rec-1, which serves as a specific receptor for the ecotropic murine leukemia viruses (MuLV-E) (Albritton *et al.*, 1989; Kim *et al.*, 1991; Wang *et al.*, 1991a,b); and the *GLVR1* gene product, which serves as a receptor for gibbon ape leukemia virus (GALV) (O'Hara *et al.*, 1990), the homologous simian sarcoma-associated virus (SSAV), and feline leukemia virus subgroup B (FeLV-B) (Takeuchi *et al.*, 1992). GLVR1 also shares homology with a phosphate permease of *Neurospora crassa* (Johann *et al.*, 1992).

Picornavirus receptors include two immunoglobulin superfamily members, poliovirus receptor (PVR) (Mendelsohn *et al.*, 1989; Nobis *et al.*, 1985) and ICAM-1, which is a specific cellular entry molecule for the major group of human rhinoviruses (Greve *et al.*, 1989; Marlin *et al.*, 1990; Staunton *et al.*, 1989). In addition, a member of the integrin family, VLA-2, is a receptor for echovirus 1 (Bergelson *et al.*, 1992). Two coronaviruses, transmissible gastroenteritis virus (TGEV) and the human coronavirus 229E (HCV-229E), use aminopeptidase N, which is a metalloprotease, as a specific receptor (Delmas *et al.*, 1992; Yeager *et al.*, 1992). Another coronavirus, mouse hepatitis virus (MHV), enters

TABLE I
Proteins as Viral Receptors[a]

Virus family	Virus	Receptor	Receptor function	Natural ligand	Evidence	Ref.
Retroviridae	HIV-1 HIV-2 SIV	CD4	TCR coreceptor Ig superfamily member	MHC II antigens	RR aRa sRb	Clapham et al. (1989); Dalgleish et al. (1984); Klatzmann et al. (1984); Maddon et al. (1986)
	MuLV-E	ecoR (Rec-1)	Basic amino acid transporter	—	RR	Albritton et al. (1989); Wang et al. (1991b)
	GALV SSAV FeLV-B	GLVR1	Phosphate permease (?)	—	RR	O'Hara et al. (1990); Takeuchi et al. (1992)
Picornaviridae	Poliovirus Type 1 Type 2 Type 3	PVR	Ig superfamily member	?	RR aRa sRb	Kaplan et al. (1990); Mendelsohn et al. (1989); Nobis et al. (1985)
	Major group of human rhinoviruses	ICAM-1	Cell adhesion molecule Ig superfamily member	LFA-1 (CD11a/CD18) Mac-1 (CD11b/CD18) (Leukocyte integrins)	RR aRa sRb	Greve et al. (1989); Marlin et al. (1990); Staunton et al. (1989)
	Echovirus 1	VLA-2	Cell adhesion molecule	Collagen and laminen	RR aRa	Bergelson et al. (1992)

(continued)

TABLE I *(Continued)*

Virus family	Virus	Receptor	Receptor function	Natural ligand	Evidence	Ref.
Caronaviridae	MHV	mmCGM1 (MHVR$_1$) mmCGM2	Integrin family member Carcinoembryonic antigens Ig superfamily member	?	rr aRa	Dveksler *et al.* (1991); Williams *et al.* (1991); Yokomori and Lai (1992)
	TGEV HCV-229E	Aminopep- tidase N	Metalloprotease	—	RR aRa	Delmas *et al.* (1992); Yeager *et al.* (1992)
Togaviridae (alphaviruses)	Sindbis virus	High-affinity laminin receptor	Cell adhesion molecule	Laminin	RR aRa	Wang *et al.* (1992)
Herpesviridae	EBV	CR2 (CD21)	Receptor for comple- ment fragments Member of a family of proteins containing short consensus repeats (SCRs)	C3dg and iC3b (complement fragments) IFN-α	RR aRa sRb	Ahearn *et al.* (1988); Fingeroth *et al.* (1984); Moore *et al.* (1991)
Papovaviridae	SV40	MHC I antigens	Antigen presentation Ig superfamily member	TCR + CD8	RR aRa	Atwood and Norkin (1989); Breau *et al.* (1992)

[a]RR, receptor reconstitution; aRa, anti-receptor antibody blocking; sRb, soluble receptor blocking of infectivity; Ig, immunoglobulin.

cells through either of two carcinoembryonic antigens (CEA), mmCGM1 (MHVR$_1$) or mmCGM2, which are members of the immunoglobulin superfamily (Dveksler et al., 1991; Williams et al., 1991; Yokomori and Lai, 1992).

The major receptor on mammalian cells for Sindbis virus, an alphavirus from the Togaviridae family, appears to be the high-affinity laminin receptor (Wang et al., 1992), an important cell adhesion molecule. The receptor for Epstein–Barr virus (EBV), an oncogenic herpesvirus associated with infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma, is the complement receptor 2 (CR2, CD21) (Ahearn et al., 1988; Fingeroth et al., 1984; Moore et al., 1991). CR2 is also a specific receptor for the C3dg and iC3b complement fragments (Cooper et al., 1988) and can specifically bind interferon α (IFN-α) (Delcayre et al., 1991). CR2 is a member of a family of proteins that contain short consensus repeats (SCRs) in their extracellular amino-terminal domain (Cooper et al., 1988), and is likely to be the EBV receptor on both human epithelial cells (Birkenbach et al., 1992) as well as B cells (Cooper et al., 1988). Finally, the well-characterized papovavirus, simian virus 40 (SV40), appears to require MHC class I antigens at the cellular surface for efficient viral entry (Atwood and Norkin, 1989; Breau et al., 1992).

Binding between virion and cell surface receptor provides the initial physical association required for entry. Viruses may enter cells by either receptor-mediated endocytosis, generally through clathrin-coated vesicles (other endocytic mechanisms also exist), or by direct fusion of the viral envelope with the cell membrane (Marsh and Helenius, 1989). In the former situation, virions are delivered to endosomes, where acidic conditions facilitate conformational changes in viral envelope proteins, permitting fusion of viral and endosomal membranes. This is followed by the entry of viral nucleic acid into the cytoplasm. Retrovirus penetration into permissive cells occurs by membrane fusion in either an acid-dependent or pH-independent fashion (Marsh and Helenius, 1989); the latter is characteristic of HIV-1 (McClure et al., 1988; Stein et al., 1987).

II. RETROVIRAL INTERFERENCE/SUPERINFECTION IMMUNITY

Cells that are productively infected with a retrovirus are resistant to superinfection by the same virus, or by viruses that possess envelope glycoproteins that bind to the same receptor (reviewed in Weiss, 1985). This phenomenon is termed *retroviral interference* or *superinfection immunity* and is caused by blocking of the cell receptor. Interfer-

ence has commonly been studied *in vitro*, through the use of pseudotypes of vesicular stomatitis virus (VSV) or retroviral vectors (carrying drug resistance genes) that bear retroviral envelope glycoproteins. Infection by such pseudotypes is blocked in cells that are infected by retroviruses that use the same receptor as that to which the pseudotypes would bind, thus enabling patterns of receptor interference to be identified. Although retroviral interference has mostly been studied *in vitro*, it has been reported that resistance may also occur *in vivo* in a mouse model involving Friend leukemia virus (Mitchell and Risser, 1992). Retroviruses have been classified into different receptor interference groups on the basis of their pattern of superinfection interference (Sommerfelt and Weiss, 1990; Weiss, 1985).

One classification of mammalian retroviruses has led to the identification of eight receptor interference groups in human cells (Sommerfelt and Weiss, 1990) and the potential existence of eight distinct retroviral receptors (Table II). The first receptor group is that with the greatest diversity and includes all known exogenous type D simian retroviruses, for example, simian retrovirus (SRV) serotypes 1–5, which infect Asian macaques, two endogenous D-type simian retroviruses that is, squirrel monkey retrovirus (SMRV) and langur endogenous retrovirus (PO-1-Lu), and two endogenous type C viruses of feline (RD114 strain) and simian (baboon endogenous virus or BaEV) origin. Data based on cross-interference patterns have shown that two avian retroviruses, that is, spleen necrosis virus (SNV) (Kewalramani *et al.*, 1992) and reticuloendotheliosis virus strain A (REV-A) (Koo *et al.*, 1992), also belong to receptor interference group 1 and are therefore likely to share a common receptor on human cells with the simian retroviruses (Table II). Use of human–rodent somatic cell hybrids (Schnitzer *et al.*, 1980; Sommerfelt *et al.*, 1990) showed that the gene that encodes the cellular receptor for this group was localized on human chromosome 19. The identity of this receptor molecule remains to be established.

Amphotropic murine leukemia viruses (MLV-A), which are capable of infecting murine and heterologous cells, did not cross-interfere with other mammalian retroviruses, and thus constitute a second receptor interference group. Endogenous xenotropic murine leukemia viruses (MLV-X) can productively infect only cells of heterologous species, do not cross-interfere with MLV-A or other mammalian retroviruses, and constitute receptor group 3. Thus, MLV-A and MLV-X most likely utilize different receptors than those of the ecotropic murine leukemia viruses (MLV-E); the latter is a basic amino acid transporter (Albritton *et al.*, 1989; Kim *et al.*, 1991; Wang *et al.*, 1991a) (Table I).

Infection of cell lines with the exogenous feline leukemia virus subgroup C did not interfere with superinfection by any other mammalian

TABLE II
RECEPTOR INTERFERENCE GROUPS[a]

Receptor group/ receptor name	Virus	Full name and strain	Type	Exogenous or endogenous origin
1	SRV-1	Simian retrovirus type 1	D	Exo
	SRV-2	Simian retrovirus type 2	D	Exo
	SRV-3 (MPMV)	Mason–Pfizer monkey virus	D	Exo
	SRV-4	Simian retrovirus type 4	D	Exo
	SRV-5	Simian retrovirus type 5	D	Exo
	PO-1-Lu	Langur endogenous retrovirus	D	Endo
	SMRV	Squirrel monkey retrovirus	D	Endo
	BaEV	Baboon endogenous retrovirus	C	Endo
	RD114	Cat endogenous retrovirus RD114	C	Endo
	REV-A	Reticuloendotheliosis virus-A	C	Exo
	SNV	Spleen necrosis virus	C	Exo
2	MLV-A	Amphotropic murine leukemia virus	C	Exo
3	MLV-X	Xenotropic murine leukemia virus	C	Endo
4	FeLV-C	Feline leukemia virus subgroup C	C	Exo
5 (GLVR1)	FeLV-B	Feline leukemia virus subgroup B	C	Exo
	SSAV	Simian sarcoma associated virus	C	Exo
	GALV	Gibbon ape leukemia virus	C	Exo
6	HTLV-I	Human T cell leukemia virus type 1	C	Exo
	HTLV-II	Human T cell luekemia virus type 2	C	Exo
	ChTLV	Chimpanzee T lymphotropic virus	C	Exo
	STLV	Simian T lymphotropic virus	C	Exo
7	BLV	Bovine leukemia virus	C	Exo
8 (CD4)	HIV-1	Human immunodeficiency virus type 1	Lenti	Exo
	HIV-2	Human immunodeficiency virus type 2	Lenti	Exo
	SIVmac	Simian immunodeficiency virus (macaque)	Lenti	Exo
	SIVsmm	Simian immunodeficiency virus (sooty mangabey)	Lenti	Exo
	SIVagm	Simian immunodeficiency virus (African Green monkey)	Lenti	Exo

[a] Adapted from Sommerfelt and Weiss (1990).

retrovirus, establishing a fourth category of receptor. Gibbon ape leukemia virus (GALV) and the closely related simian sarcoma associated virus (SSAV), as well as feline leukemia virus subgroup B (FeLV-B) cross-interfere with one another and belong to receptor group 5. The gene that encodes this receptor, termed *GLVR1*, has been isolated and shown to resemble a phosphate permease (Johann *et al.*, 1992; O'Hara *et al.*, 1990; Takeuchi *et al.*, 1992) (Table I).

Interference patterns have established that a common receptor is shared by the human T cell leukemia viruses (HTLV-I and HTLV-II) and the related chimpanzee and simian T lymphotropic viruses (ChTLV and STLV) (receptor group 6). The gene encoding this receptor is on human chromosome 17 (Sommerfelt *et al.*, 1988). The bovine leukemia virus (BLV) utilizes a different receptor from that of the group 6 T cell leukemia viruses and is the sole known member of receptor group 7, in spite of having a genetic structure similar to HTLV-I, including the two regulatory genes *tax* and *rex*. The last receptor group (group 8) is that which binds to CD4 and is exemplified by the human and simian immunodeficiency viruses (HIV-1, HIV-2, SIVmac, SIVsmm, SIVagm) (Sattentau, 1988) (Table II).

Retroviral envelope glycoproteins are a key determinant in both interference and cell killing. Cell lines that were engineered to express avian REV envelope proteins became refractory to infection by viruses of the REV group (Delwart and Panganiban, 1989; Federspiel *et al.*, 1989). Cell lines that expressed a mutant SNV *env* gene, the product of which lacked the carboxy end of the transmembrane protein (TM) and was blocked in the endoplasmic reticulum (ER), were also resistant to infection (Delwart and Panganiban, 1989). In HIV-1-infected cells or in cells coexpressing CD4 and the HIV-1 envelope glycoproteins, CD4 was found to be associated with gp160 (the envelope precursor), causing their mutual retention in the ER (Bour *et al.*, 1991; Crise *et al.*, 1990; Kawamura *et al.*, 1989; Stevenson *et al.*, 1988). Therefore, superinfection immunity is the likely consequence of the sequestering of cellular receptors by viral envelope glycoproteins in the ER.

Superinfection interference may sometimes be separated from infectiousness. Cells that stably expressed an MuLV-E mutant, carrying a deletion in the membrane-spanning region and the cytoplasmic tail of the TM protein that affected viral infectivity, but not processing or incorporation of envelope proteins, were resistant to superinfection (Granowitz *et al.*, 1991). The resistance of some animals to certain strains of retroviruses is attributable to expression of envelope proteins by defective endogenous proviruses, probably through intracellular receptor–envelope interactions (Temin, 1988).

Failure to establish or delay establishment of retroviral interference

may lead to massive reinfection and cell death, accompanied by accumulation of unintegrated viral DNA (Donahue et al., 1991; Haase et al., 1982; Pauza et al., 1990; Temin, 1988; Weller et al., 1980). env gene determinants play important roles in retrovirus-induced cytopathicity (Donahue et al., 1991; Dorner and Coffin, 1986), and cytopathic viruses are commonly unable to establish superinfection interference (Donahue et al., 1991). This may be attributable to a slower rate of synthesis and processing of viral envelope precursors (Poss et al., 1990) or to the reduced efficiency with which such molecules associate intracellularly with the receptor (Temin, 1988). Additional factors that may slow superinfection interference include rapid receptor recycling, and rapid viral replication, which enables superinfection to occur before establishment of interference (Temin, 1988). Finally, efficient displacement of envelope receptor-associated glycoprotein by incoming viral envelope glycoprotein, and slower kinetics of interference due to high-level expression of receptors, may equally facilitate superinfection (Dorner and Coffin, 1986).

Addition of neutralizing antibodies, anti-CD4 antibodies, or 3'-azido-3'-deoxythymidine (AZT) to acutely infected T cell lines commonly leads to reduction in rates of accumulation of unintegrated viral DNA (Bergeron and Sodroski, 1992; Pauza et al., 1990; Robinson and Zinkus, 1990). T cell lines that were engineered to express the HIV-1 envelope gene had reduced levels of cell surface CD4, due to complex formation between CD4 and gp160 (Stevenson et al., 1988). Superinfection by HIV-1 of such envelope-expressing cells was noncytopathic and persistent in nature. In contrast, infection of parental T cell lines was accompanied by accumulation of unintegrated viral DNA and cytopathicity. Inhibition of superinfection was associated with both a diminution in accumulation of unintegrated viral DNA and syncytium formation (an early cytopathic effect), leading to persistent infection (Pauza et al., 1990; Stevenson et al., 1988). However, a delayed cytopathic effect, termed single-cell lysis, was not prevented by inhibition of superinfection, because the latter occurred in the absence of unintegrated viral DNA accumulation and when CD4 had been completely depleted from the cell surface (Bergeron and Sodroski, 1992; Stevenson et al., 1988).

Macrophage-tropic HIV-1 variants from brain failed to modulate cell surface CD4 expression after infection. In this instance, both the capacity for CD4 modulation and macrophage tropism were mapped to the envelope gene (Cheng-Mayer et al., 1990). The failure of CD4 downmodulation in brain may be related to superinfection and accumulation of unintegrated viral DNA in the brains of patients with HIV-1 encephalitis (Pang et al., 1990).

III. STRUCTURE AND FUNCTION OF CD4 AND p56^{lck}

A. *CD4–MHC II Interaction*

CD4 is a transmembrane glycoprotein of approximately 55 kDa, predominantly expressed on the surface of certain subsets of mature T lymphocytes and thymocytes. CD4$^+$ T lymphocytes specifically recognize foreign antigens as peptides presented by self major histocompatibility class II (MHC II) molecules (reviewed in Bierer *et al.*, 1989; Littman, 1987; Parnes, 1989). Studies in which fibroblasts were engineered to express CD4 showed that this molecule confered the ability to aggregate class II MHC-expressing B cells (Doyle and Strominger, 1987), suggesting a physical interaction between the two molecules. This interaction was shown to be important in T cell activation because effector functions of CD4$^+$ T cells were inhibited with anti-CD4 monoclonal antibody (MAb) (Biddison *et al.*, 1982; Swain *et al.*, 1984; Wilde *et al.*, 1983). In addition, cell lines transfected with CD4 expression vectors were shown to secrete enhanced levels of interleukin 2 (IL-2), in the presence of cells that expressed human MHC II antigens; such T cell activation was abrogated by antibodies against either CD4 or MHC II determinants (Gay *et al.*, 1987; Sleckman *et al.*, 1987). Therefore, the CD4–MHC II interaction not only increases the avidity between CD4$^+$ T cells and antigen-presenting cells (APCs), but also plays a role in signal transduction leading to T cell activation (reviewed in Bierer *et al.*, 1989; Janeway, 1992; Parnes, 1989).

Cross-linking of CD4 molecules, in a manner independent of the T cell receptor (TCR) at the T cell surface, and in the absence of MHC class II antigens, inhibited T cell activation by inducing a negative signal (Haque *et al.*, 1987; Wassmer *et al.*, 1985). This inhibitory effect was tightly associated with p56^{lck} activity, a subject to be examined in the next section. CD4 probably binds to the same MHC molecule on an APC as does the TCR during T cell activation (reviewed in Janeway, 1992). This association between CD4 and the TCR enhanced signal transduction, suggesting that CD4 may be a coreceptor as well as an accessory molecule involved in cellular adhesion (reviewed in Janeway, 1992).

B. *Role of CD4 and p56^{lck} in Thymic Development*

CD4 is not only necessary for T cell activation but also contributes to the shaping of the T cell repertoire during thymic development (Robey and Axel, 1990). CD4$^+$ and CD8$^+$ mature T cells develop from CD4$^-$CD8$^-$ (double-negative) immature thymocytes that lack surface

TCR expression. Differentiation of these cells into CD4+CD8+ (double-positive) thymocytes is preceded by rearrangement and expression of the *TCR* genes (α and β), leading to low-level surface representation of TCR. These double-positive T cells then lose either CD4 or CD8 expression, to become either CD4+CD8− or CD4−CD8+ (single positive) with high levels of surface TCR (von Boehmer, 1988).

Transgenic mouse models have revealed that the MHC specificity of TCRs, that is, class II or class I restricted, determines the CD4/CD8 phenotype of peripheral T cells (von Boehmer, 1990). Surface TCR expression was shown to increase in freshly explanted double-positive thymocytes, but could be blocked by CD4-mediated signals, for example, multivalent cross-linking of MAbs bound to CD4 or MHC II engagement of CD4. Thus, signaling through CD4 in double-positive thymocytes resulted in diminished cell surface expression of TCR, suggesting that negative signaling had occurred (Nakayama *et al.*, 1990).

CD4 coding sequences, under the transcriptional control of the proximal *lck* promoter or CD2 regulatory elements, were used to generate transgenic mice, in which CD8+ peripheral T cells equally expressed CD4. CD4+CD8+ T cells from such animals proliferated in response to both allogeneic class I and class II MHC antigen (Robey *et al.*, 1991; Teh *et al.*, 1991): such dual reactivity suggested that the ability of T cells to react to allogeneic class II MHC was determined by CD4. The proximal *lck* promoter, used to generate the transgenic mice, lies immediately 5′ to the p56^{lck} coding region and is highly active in the thymus, contributing to high-level CD4 expression on thymocytes (Reynolds *et al.*, 1990). The distal *lck* promoter, which is located far upstream of the proximal promoter, is active in both thymocytes and mature T cells (Wildin *et al.*, 1991). Overexpression of CD4 in thymocytes of transgenic animals dramatically inhibited positive selection, as assessed by lower levels of CD8+ thymocytes bearing a specific TCR in comparison to control animals (Teh *et al.*, 1991). These results demonstrated that altered CD4 levels in the thymus may influence the shape of the T cell repertoire.

Thymocytes from transgenic mice that expressed a TCR specific for MHC I and a hybrid CD8/CD4 molecule, composed of an extracellular CD8 and both a transmembrane domain and cytoplasmic tail of CD4, differentiated into mature T cells that expressed CD4 (Seong *et al.*, 1992). Thus, the cytoplasmic tail of CD4, which associates with p56^{lck}, mediates a signal that directs differentiation of immature thymocytes to the CD4 lineage. In contrast to the importance of p56^{lck} in T cell activation (see Section III,D below), it appears that the process of clonal deletion of immature thymocytes may be independent of p56^{lck} in tissue culture (Nakayama and Loh, 1992). These results suggest that the

signal transduction pathway that leads to clonal deletion by apoptosis is distinct from that of T cell activation.

Knock-out technology, causing disruption of the *CD4* gene, generated mice that no longer expressed cell surface CD4 and that displayed reduced helper cell activity for antibody responses. However, the development of CD8^{+} T cells and CTL activity against virally (e.g., vaccina) infected cells was not affected (Rahemtulla *et al.*, 1991). Mice in which the gene encoding p56[*lck*] was disrupted had severely reduced numbers of both peripheral T cells and double-positive (CD4^{+}CD8^{+}) thymocytes, as well as pronounced thymic atrophy (Molina *et al.*, 1992). These reports demonstrate the importance of the CD4 and p56[*lck*] molecules in T cell development.

C. D1 and D2 Domains of CD4: Interactions with gp120 and MHC II

In addition to its role in T cell activation and thymic development, CD4 is also a high-affinity receptor for HIV-1 (Sattentau and Weiss, 1988) (Table I). The CD4 molecule is a member of the immunoglobulin (Ig) gene superfamily and consists of four external Ig-like domains (370 amino acids) (D1–4 also referred to as V1–4), a transmembrane region (26 amino acids), and a cytoplasmic tail (38 amino acids) (Littman, 1987; Maddon *et al.*, 1985), which is associated with the p56[*lck*] protein tyrosine kinase (reviewed in Bolen and Veillette, 1989; Rudd, 1990) (Fig. 1). On the basis of sequence and structural homology between the CD4 D1 domain and the immunoglobulin κ light chain variable domain (V), three complementarity-determining regions (CDR1–3) have been described in D1: CDR1 (amino acid positions 18–27), CDR2 (amino acids 42–49), and CDR3 (amino acids 85–97). Domains D1 and D2 contain residues involved in MHC II binding.

Binding of HIV-1 gp120 is mediated by D1 (Capon and Ward, 1991; Clayton *et al.*, 1989; Fleury *et al.*, 1991; Lamarre *et al.*, 1989; Sattentau, 1988). Mutagenesis of this region has further shown the importance in this respect of residues that comprise and surround the CDR2-like region (amino acids 40–55) (reviewed in Capon and Ward, 1991; Sattentau, 1988).

Although the complete molecular structure of CD4 has not been defined, crystallographic studies have suggested that CD4 forms a rodlike structure about 125 Å in length and 25–30 Å wide (Kwong *et al.*, 1990). Two reports have determined the structure of a soluble fragment of CD4, consisting of the two amino-terminal domains, D1 and D2, that is, about half the complete molecule (Ryu *et al.*, 1990; Wang *et al.*, 1990). Both D1 and D2 resemble Ig domains (antiparallel β strands folding to form two β sheets held together by a disulfide bond and hydrophobic interactions), consisting of nine and seven strands, respec-
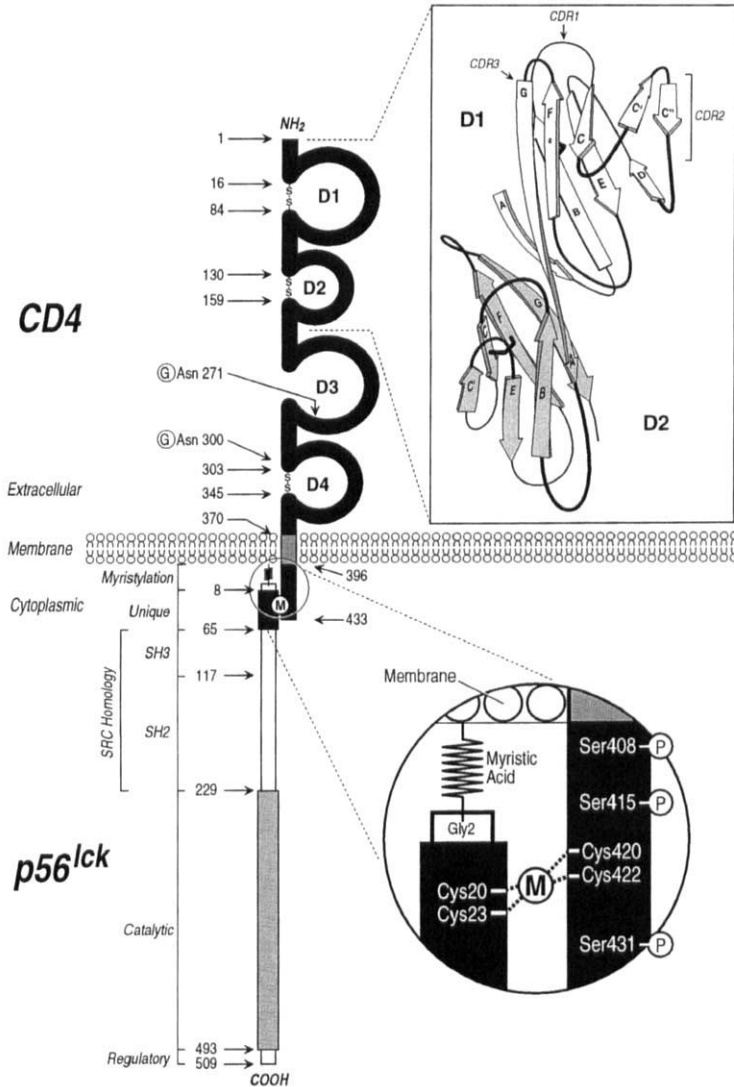
FIG. 1. Schematic representation of the CD4–p56*lck* complex. *Top (inset):* the D1 and D2 domains of CD4 as determined by crystallographic studies. [Reprinted with permission from *Nature (London)*, ref. from Wang *et al.*, 1990. Copyright 1990 Macmillan Magazines Limited.] *Bottom (inset):* Putative intermolecular association between CD4 and p56*lck*.

tively (Fig. 1, top inset). The D1 domain contains two antiparallel β sheets; strands B, D, and E make up one of these, whereas strands A, C, C′, C″, F, and G make up the other. The disulfide bond is between strands B and F (on opposing sheets). Strands A, B, and E make up one β sheet in D2, whereas strands C, C′, F, and G compose the other. The D2 is

truncated in comparison with Ig variable domains. An unusual disulfide bond in D2 is present between strands C and F in the same sheet.

D1 and D2 are associated by means of a long β strand that forms the last strand (G) of the former while continuing to become the first (A) of the latter; this confers rigidity to the amino terminus of CD4. Strands C', C", and D form a loop in D1, which encompasses the CDR2-like region, essential for binding of gp120. This loop is longer than that in Ig and forms a prominent ridge composed of the anti-parallel C' and C" strands (Fig. 1, top inset). Additional residues involved in gp120 binding lie in the adjacent D strand, suggesting that gp120 may have a complementary groove on its surface that interacts with this ridge.

The binding site on CD4 for MHC II determinants is more complex than that for gp120 and involves residues in D2, in addition to D1 (Clayton et al., 1989; Fleury et al., 1991; Lamarre et al., 1989). Mutations that abolish binding of gp120, such as at the hydrophobic residue Phe-43 on the C" strand, do not necessarily alter the general structure of CD4 but can affect binding of MHC II (Arthos et al., 1989; Bowman et al., 1990; Moebius et al., 1992). Replacement of four residues (amino acids 39 to 43) by an equivalent segment, derived from murine L3T4, yielded a modified CD4 structure that could still bind MHC II, but not gp120 (Lamarre et al., 1989).

Other residues in D1 that are important for binding of both MHC II and gp120 include Pro-48, Lys-50, and Leu-51, located in CDR2 (Clayton et al., 1989; Lamarre et al., 1989). Deletion of residues 42–47 or 43–49 caused conformational changes in CD4 (Bowman et al., 1990) that led to the disruption of both MHC II and gp120 binding in one study (Bowman et al., 1990) and gp120 binding in another (Fleury et al., 1991); both deletions removed the Phe-43 residue, critical for gp120 binding.

D1 residues at positions 19 (CDR1), 89 (CDR3), and D2 residue 165 are critical for MHC II binding (Fleury et al., 1991). The lack of effect of the residue 43–49 deletion on MHC II binding suggests that gp120 and MHC II may contact CD4 on opposite sides (Fleury et al., 1991): gp120 may require CDR2 residues and MHC II involvement may require CDR1 and CDR3 of D1 and the FG loop of D2 (Fig. 1).

Mutations in residues (amino acids 54–57) in the D strand were also shown to affect CD4 structure and both MHC II and gp120 binding (Piatier-Tonneau et al., 1991); an oligopeptide derived from CD4 amino acids 54–57 inhibited antigen-specific, MHC II-restricted T cell responses (Mazerolles et al., 1988). Other residues in D1 of CD4 are also involved in binding of gp120 and MHC II, suggesting alternatively that the binding sites for these molecules may overlap (Bowman et al., 1990; Moebius et al., 1992).

Residue Glu-87 located in CDR3 of D1 may play a role in HIV-

induced syncytium formation, as this effect was abolished following replacement of this amino acid by the equivalent chimpanzee residue Gly-87 (Camerini and Seed, 1990). The converse substitution conferred to the chimpanzee CD4 the ability to participate in HIV-associated syncytium formation. In addition to binding gp120 and MHC II, CD4 can also bind Ig. Residues 21–38 of the CC′ loop in D1 were shown to be important in this latter association (Lennert et al., 1990).

Monoclonal antibodies against the D2 and D3 domains of CD4 can block both HIV-1 infection and syncytium formation (Burkly et al., 1992; Healy et al., 1990; Moore et al., 1992). However, these MAbs are unable to impede the primary attachment of gp120 to CD4. This suggests that MAb binding to D2 or D3 may interfere with the postbinding conformational changes required for membrane fusion, in either CD4 or the viral envelope proteins. Removal of two N-linked glycosylation sites, that is, asparagines at positions 271 and 300 in D3 and D4 (Fig. 1), did not prevent infection (Bedinger et al., 1988). Thus, CD4 may also be involved in postbinding steps that during the process of viral entry are independent of N-linked glycosylation and that may include membrane fusion.

The binding site of CD4 on MHC II molecules has been mapped to the membrane-proximal β2 domain of the β chain (Cammarota et al., 1992; König et al., 1992). This domain is structurally similar to the α3 domain of MHC I heavy chains, which has been shown to bind to CD8 (Salter et al., 1990).

D. CD4–p56^{lck} Association

CD4 is associated noncovalently through its cytoplasmic tail with the p56^{lck} protein tyrosine kinase (PTK), a member of the Src family of PTKs (reviewed in Bolen and Veillette, 1989; Rudd, 1990; Ettehadieh, et al., 1992). The structural organization of p56^{lck} and its association with CD4 are depicted in Fig. 1. The first eight amino acids at the N terminus of p56^{lck} are important for myristylation and stable membrane association. p56^{lck} is associated with the inner face of the cytoplasmic membrane through a myristic acid group covalently linked to an N-terminal glycine residue at position 2 (Fig. 1, bottom inset). Each member of the Src family possesses a unique amino-terminal region (amino acids 8–65 in p56^{lck}) believed to govern specific interactions with other cellular proteins. This is the region of greatest sequence diversity among Src family members, and is followed by two SH (Src homology) regions termed SH3 (residues 65–117 in p56^{lck}) and SH2 (residues 117–229 in p56^{lck}). The latter two motifs are shared with other nonreceptor PTKs such as those of the fes/fps (SH2 only) and abl gene families. SH sequences are also found in other proteins including

phospholipase Cγ, GTPase-activating protein (GAP), and the Gag–Crk fusion product of an avian retrovirus (reviewed in Bolen *et al.*, 1991; Cantley *et al.*, 1991).

The SH region is important in the regulation of Src family enzymatic activity. The SH3 domain may be important in the localization of PTKs to cytoskeletal components near the cytoplasmic membrane (Bolen, 1991; Bolen *et al.*, 1991). The SH2 domain is believed to interact with the regulatory phosphotyrosine located at the carboxy terminus of Src PTKs (Tyr-505 in p56^{lck}). The region of highest sequence homology among Src family members resides in the catalytic or kinase domain (also referred to as SH1), which represents the major portion of the carboxy terminus situated between amino acid 229 and 493 in p56^{lck}. The catalytic domain also contains the ATP-binding site, which is centered around a common lysine residue (Lys-273 in p56^{lck}), and the autophosphorylation site (Tyr-394 in p56^{lck}), which is thought to play an important role in catalytic activity and to favor interactions with potential substrates.

The final 16 carboxy-terminal amino acids (positions 493–509) comprise the regulatory domain of p56^{lck}, which is important for basal PTK activity. This region has a central conserved tyrosine (Tyr-505 in p56^{lck}) that is normally phosphorylated. Mutation of this Tyr residue results in constitutive activation of PTK activity (Abraham *et al.*, 1991) associated with enhanced transforming potential; this suggests that the phosphotyrosine residue of the regulatory domain may be an inhibitor of enzymatic activity. Hence, dephosphorylation of this Tyr residue may be a mechanism of regulative PTK activity (Bolen, 1991; Bolen *et al.*, 1991; Cantley *et al.*, 1991).

Studies on CD4–VSV G (vesicular stomatitis virus protein G) and Src–Lck hybrid molecules, as well as on deletion mutants of CD4 and p56^{lck}, have shown that the cytoplasmic tail of CD4 and the amino-terminal unique domain of p56^{lck} (i.e., 38 C-terminal residues of CD4 plus the first 32 N-terminal residues of p56^{lck}) are sufficient for interaction of the latter 2 proteins (Shaw *et al.*, 1989). Furthermore, site-directed mutagenesis has established that two closely positioned cysteine residues in the cytoplasmic tail of CD4 (positions 420 and 422) are critical for binding to p56^{lck} (Shaw *et al.*, 1990) (Fig. 1, bottom inset). Two similarly positioned cysteine residues in murine L3T4 are also important in p56^{lck} binding (Turner *et al.*, 1990). Several cysteines in the unique N terminus of p56^{lck} are also involved in association with CD4. Specifically, cysteines at positions 3 and 5, and a glycine at position 2 (due to its linkage to myristic acid), are important in localizing p56^{lck} to the cytoplasmic membrane (Turner *et al.*, 1990). A second pair of cysteines at positions 20 and 23 are essential for association with

CD4 (Shaw *et al.*, 1990; Turner *et al.*, 1990) (Fig. 1, bottom inset). This CD4–p56^{lck} association can be inhibited by alkylating agents that interact with free sulfhydryls; hence, free cysteines rather than interchain disulfide bonds are probably involved (Barber *et al.*, 1989; Shaw *et al.*, 1990). This also suggests that CD4–p56^{lck} interactions may involve a metal ion (Fig. 1).

E. *Role of Cytoplasmic Tail of CD4 in Endocytosis and HIV-1 Entry*

CD4 endocytosis can be induced by phorbol esters such as phorbol 12-myristate 13-acetate (PMA), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and phorbol 12,13-dibutyrate (PDB) (Acres *et al.*, 1986; Hoxie *et al.*, 1986b), which are all potent activators of protein kinase C (PKC). Cell surface CD4 internalization is accompanied by phosphorylation of serine residues in the cytoplasmic tail of CD4 (Shin *et al.*, 1990) and dissociation of p56^{lck} from CD4 (Hurley *et al.*, 1989). The intracellular cytoplasmic tail of CD4 is crucial for internalization because CD4 mutants with deleted cytoplasmic tails fail to internalize following phorbol ester treatment (Bedinger *et al.*, 1988; Maddon *et al.*, 1988).

Serine phosphorylation by PKC plays an important role in initiating endocytosis. First, PKC inhibitors, such as H7, inhibit CD4 internalization (Maddon *et al.*, 1988; Munck Peterson *et al.*, 1992). Second, CD4 mutants in which Ser-408 (or the equivalent Ser-406 in mouse CD4), -415, and -431 were altered (Fig. 1, bottom inset) were impaired in their ability to internalize in response to treatment with phorbol esters (Bedinger *et al.*, 1988; Glaichenhaus *et al.*, 1991; Maddon *et al.*, 1988; Shin *et al.*, 1990), with the highest degree of inhibition reported in cells mutated at all three residues (Shin *et al.*, 1990). Nor was the mutated protein phosphorylated following phorbol ester treatment (Shin *et al.*, 1990). In addition, a cytoplasmic deletion mutant of CD4, in which the C-terminal residues 418–433 were deleted (Fig. 1), was capable of internalization following treatment with phorbol esters. Thus, the membrane-proximal region of the cytoplasmic tail of CD4, containing residues 396–417 and forming a potential α helix, is sufficient to permit CD4 endocytosis (Shin *et al.*, 1991b). Phosphorylation of Ser-408 may be necessary to initiate disruption of the CD4–p56^{lck} complex, which precedes phorbol ester-induced CD4 internalization (Hurley *et al.*, 1989; Sleckman *et al.*, 1992). In addition, cytoplasmic tail deletion mutants of CD4 were found to internalize more frequently than wild-type CD4, which may be excluded from coated pits because of its association with p56^{lck} (Pelchen-Matthews *et al.*, 1991), suggesting that p56^{lck} may inhibit CD4 endocytosis.

CD4 molecules with either individual serine mutations or the triple

mutation remained competent to associate with p56^{lck} (Glaichenhaus *et al.*, 1991), suggesting that these serines are not important in this regard. However, cells that expressed the triple serine mutation, but not individual mutations, responded only weakly to antigen–MHC II stimulation, as assessed by lower levels of IL-2 production. Thus, a mutation at Ser-408 can impair CD4 internalization but neither re-sponsiveness to antigenic stimulation nor association with p56^{lck}, sug-gesting that two independent functional regions are located within the cytoplasmic tail of CD4. These are a membrane-proximal region (resi-dues 396 to 417, including Ser-408) important for both dissociation of CD4–p56^{lck} and CD4 endocytosis, and a region centered around Cys-420 and -422 essential for p56^{lck} association (Fig. 1).

Cytoplasmic tail deletion mutants or Ser-408 mutants of CD4 can serve as efficient HIV receptors, in spite of their failure to be inter-nalized (Bedinger *et al.*, 1988; Maddon *et al.*, 1988). Thus, HIV may enter cells by a mechanism independent of receptor-mediated endo-cytosis. Coincidentally, the kinetics of viral replication were delayed in cells that expressed CD4 molecules containing cytoplasmic tail dele-tions (Poulin *et al.*, 1991). In addition, CD4 molecules anchored to the cell membrane by glycophospholipid bonds served as efficient HIV-1 receptors, suggesting that both the cytoplasmic tail, as well as the transmembrane domain of CD4, were dispensable for infection (Dia-mond *et al.*, 1990; Jasin *et al.*, 1991; Kost *et al.*, 1991). These CD4 molecules were chimeras in which the extracellular domain of CD4 was fused to the C-terminal regions of either decay-accelerating factor (DAF) (Kost *et al.*, 1991) or lymphocyte function-associated antigen 3 (LFA-3) (Diamond *et al.*, 1990). These C-terminal segments are suffi-cient to direct glycophospholipid anchoring. In other studies, the two N-terminal domains of CD4 were fused to the mouse Thy-1 antigen (Jasin *et al.*, 1991).

HIV-1 entry was also shown to be independent of both pH (McClure *et al.*, 1988; Stein *et al.*, 1987) and CD4-related signal transduction events (Orloff *et al.*, 1991). In the former case, neutralization of endo-somal compartments with lysosomotropic agents such as the weak bases chloroquine, amantadine, and ammonium chloride as well as the carboxylic acid ionophore monensin failed to inhibit HIV-1 entry (Mc-Clure *et al.*, 1988; Stein *et al.*, 1987). H7 (PKC inhibitor), EGTA (extra-cellular calcium chelator), cyclosporin A (inhibitor of calcium/ calmodulin-dependent activation), and pertussis toxin (inhibition of G protein function) also failed to inhibit penetration of HIV into cells, suggesting a dissociation between infection and signal transduction.

Deletion of the 13 C-terminal amino acids of the CD4 cytoplasmic tail generated a molecule that was retained in the ER (Shin *et al.*,

1990). The C terminus of this mutant is 416 Ser-Glu-Lys-Lys-Thr-Ser 421. Site-directed mutagenesis showed that the ER retention property of this molecule was conferred by the two lysine residues. However, changing Lys-419 or Thr-420 to the positively charged residues Arg or Lys, respectively, did not affect ER retention. The latter signal (Lys-Lys-X-X, where X is any amino acid) is functional only in the presence of a transmembrane region, unlike the ER lumenal retention signal Lys-Asp-Glu-Leu (KDEL).

F. Role of CD4–p56[lck] in T Cell Activation

Cross-linking of cell surface CD4 on murine T lymphocytes resulted in both enhanced autophosphorylation and phosphorylation of an exogenous substrate by p56[lck] (Veillette *et al.*, 1989). Cross-linking of CD4 also resulted in specific tyrosine phosphorylation of the ζ chain of the CD3 complex, associated with the TCR. The use of both anti-CD4 MAbs as well as secondary cross-linking antibody was needed to increase p56[lck] PTK activity, because monovalent fragments of the same anti-CD4 MAb did not yield this effect. Hence, the CD4–p56[lck] receptor–kinase unit is capable of signal transduction as assessed by an early event in T cell signaling, namely, tyrosine phosphorylation.

Signaling through CD4 (without involvement of the TCR–CD3 complex) resulted in an inhibitory effect termed *negative signaling* (reviewed in Janeway, 1992). This inhibitory effect, initiated by CD4 cross-linking, was also shown to be mediated by p56[lck] and correlated with p56[lck] PTK activity (Janeway, 1992; Takahashi *et al.*, 1992). Cross-linking of CD4, followed by signaling through TCRαβ resulted in the death of T cells by apoptosis (Newell *et al.*, 1990).

Cross-linking of CD4 with the TCR led to even greater levels of tyrosine phosphorylation (Abraham *et al.*, 1991; June *et al.*, 1990) and T cell activation (Owens *et al.*, 1987), suggesting that physical association between CD4 and TCR is required for this to occur. CD4 may be involved through delivery of p56[lck] to the TCR–CD3 complex. A mutated form of p56[lck] (Tyr-505→Phe) was constitutively activated in a CD4-negative murine hybridoma that expressed a defined TCR (Abraham *et al.*, 1991). Coincubation of such cells with cells that expressed appropriate combinations of MHC II plus antigen gave rise to enhanced levels of IL-2 secretion similar to those seen when CD4 was present in this murine hybridoma. Antibody-mediated aggregation of TCR–CD3 in cells expressing the Phe-505 p56[lck] mutant also led to enhanced tyrosine phosphorylation, but not to the extent seen following coaggregation of TCR and CD4 in CD4-transfected cells (Abraham *et al.*, 1991).

During T cell activation, tyrosine phosphorylation is thought to pre-
cede the breakdown of phosphoinositides (PI) into diacylglycerol and
inositol phosphates; the former is involved in the activation of protein
kinase C, whereas the latter are important in the mobilization of cyto-
solic calcium (Klausner and Samelson, 1991). The importance of p56^{lck}
in this signal transduction cascade was demonstrated in a mutant T
cell line that failed to show Ca^{2+} increases following TCR engagement,
and that was defective in tyrosine phosphorylation (Straus and Weiss,
1992). Furthermore, this cell line was defective in p56^{lck} expression.
Restoration of p56^{lck} function by gene transfer restored ability to re-
spond to TCR stimulation.

The importance of p56^{lck} in T cell activation was further documented
by use of CD4 mutants that failed to associate with the former mole-
cule. Murine cell lines that coexpressed TCRs of known antigen speci-
ficity and human or murine CD4 molecules with deleted cytoplasmic
tails were inefficient at secreting IL-2 in response to appropriate MHC
II−antigen combinations (Glaichenhaus *et al.*, 1991; Miceli *et al.*, 1991;
Sleckman *et al.*, 1988). An even greater reduction in IL-2 production
was seen in cells that expressed a glycolipid-anchored CD4 lacking
both its cytoplasmic tail and transmembrane domain, despite its abili-
ty to mediate intercellular adhesion (Sleckman *et al.*, 1991).

Failure to respond to MHC−peptide stimulation was also demon-
strated in T cells that expressed murine CD4 mutants, in which
Cys-418 and -420 of the cytoplasmic tail were changed to alanines
(Glaichenhaus *et al.*, 1991). Equivalent mutations of human CD4
Cys-420 and -422, which abolish p56^{lck} association, had the same ef-
fect (Shaw *et al.*, 1990) (Fig. 1). In addition, these cysteine mutants of
CD4 failed to associate with TCR−CD3 following treatment with spe-
cific anti-CD3 antibody that aggregated TCR−CD3, suggesting that
CD4, in the absence of p56^{lck}, does not associate with the TCR−CD3
complex and further emphasizing the importance of p56^{lck}−TCR/CD3
juxtaposition in T cell activation.

Both anti-TCR MAb binding, and antigenic stimulation in the prop-
er MHC II context, induced similar levels of activation in a murine
cellular clone that expressed a CD4 structure containing both the
double-cysteine mutation and a TCR of defined specificity (Haughn *et
al.*, 1992). In contrast, the same cellular clone when engineered to
express wild-type CD4 was activated more efficiently by MHC II−
antigen than by anti-TCR MAb. Furthermore, cross-linking of CD4
and TCR or the TCR alone at the surface of the double-cysteine mutant
led to similar levels of activation, as assessed by DNA synthesis, cal-
cium mobilization, and tyrosine phosphorylation. In contrast, clones

that expressed wild-type CD4 were minimally responsive to anti-TCR cross-linking, but had levels of activation similar to those of the double-cysteine mutant after CD4–TCR cross-linking. This shows that the CD4–p56^{lck} receptor–kinase prohibits T cell activation when not juxtaposed properly to the TCR–CD3 complex at initiation of signaling. Hence, CD4 may be able to sequester the p56^{lck} needed to couple the TCR to the CD3 complex in order for successful activation to occur. Previous reports of ζ chain tyrosine phosphorylation by p56^{lck} may be related to this process (Veillette et al., 1989). The CD4–p56^{lck} receptor–kinase complex is able to mediate signals important for T cell activation, distinct from the role of CD4 in adhesion (Doyle and Strominger, 1987).

G. Intermolecular Associations Involving p56^{lck}

p56^{lck} can also interact with the IL-2β chain and treatment of T cells with IL-2 results in p56^{lck} activation (Hatakeyama et al., 1991; Horak et al., 1991). This suggests that this PTK may also participate in IL-2-mediated signal transduction events. Because p56^{lck} is a necessary link between IL-2 receptor-binding tyrosine phosphorylation, and T cell activation, it is relevant that CD4 cross-linking renders T cells nonresponsive to IL-2, probably by sequestering the majority of intracellular p56^{lck} (Takahashi et al., 1992). Signaling through the TCR–CD3 complex is also inhibited in the absence of CD4–p56^{lck} participation (Haughn et al., 1992). The IL-2 receptor, like TCR–CD3, may thus require p56^{lck} to couple cell surface receptor-binding events to downstream signal transduction steps.

The membrane-bound CD45 phosphotyrosine phosphatase is involved in regulation of p56^{lck} PTK activity by altering the state of tyrosine phosphorylation of this enzyme. Cell lines that lack CD45 had two to three times more phosphorylation at the p56^{lck} Tyr-505 than is commonly observed (Ostergaard et al., 1989) but no p56^{lck} activation (Mustelin et al., 1989). Moreover, CD45 appeared to enhance p56^{lck} PTK activity directly in cell-free systems, an activity that was blocked by sodium orthovanadate, which inhibits phosphotyrosine phosphatase activity (Mustelin et al., 1989). These findings are consistent with the observation that phosphotyrosine-505 of p56^{lck} may be a negative regulatory element, dephosphorylation of which activates p56^{lck} and implicates CD45 in this activation. However, cross-linking of CD4 with CD45 failed to induce tyrosine phosphorylation (Ostergaard and Trowbridge, 1990), suggesting that coclustering of CD45 with CD4 leads to dephosphorylation of p56^{lck} and a reduction in p56^{lck} activity.

This may be related to dephosphorylation of phosphotyrosine-394, which is important in catalytic activity and substrate association, rather than phosphotyrosine-505, which enhances PTK activity.

The p50^{csk} PTK may also be involved in the negative regulation of p56^{lck}, as it has been shown to phosphorylate Tyr-505 specifically, a step that suppressed catalytic activity (Bergman *et al.*, 1992). p56^{lck} was shown both to phosphorylate and then associate with GTPase-activating protein (GAP) (Amrein *et al.*, 1992). p56^{lck} also associates with other molecules involved in signal transduction, including a GTP-binding protein (Telfer and Rudd, 1991), and tyrosine-phosphorylated phosphoinositide-specific phospholipase Cγ1 (PLCγ1) (through its SH2 domain) (Weber *et al.*, 1992). Substrates for p56^{lck} include both the CD3 ζ chain (Veillette *et al.*, 1989) and the Raf-1 kinase, following cross-linking of cell surface CD4 (Thompson *et al.*, 1991), and the mitogen-activated protein (MAP) kinase (p42^{mapk}) following treatment of murine CD4$^+$ T cells with immobilized anti-CD3 MAb (Ettehadieh *et al.*, 1992).

H. Consequences of gp120 Binding to CD4

Soluble gp120, like certain anti-CD4 MAbs, can inhibit T lymphocyte proliferation in response to mitogens, antigen, and anti-CD3 MAb (Corado *et al.*, 1991; Oyaizu *et al.*, 1990; see also review in Habeshaw *et al.*, 1990). This effect was dependent on the ability of gp120 to interact with CD4, and was blocked by soluble CD4 (Oyaizu *et al.*, 1990; see also review in Habeshaw *et al.*, 1990). The immunosuppressive effect of gp120 may result from inhibition of CD4–MHC II interactions through steric hindrance (Clayton *et al.*, 1989; Lamarre *et al.*, 1989; Rosenstein *et al.*, 1990). In addition, gp120 was shown to cointernalize with CD4, and concomitantly to induce p56^{lck} dissociation and a reduction in T cell responsiveness to specific antigen or anti-CD3 MAb (Cefai *et al.*, 1992).

It is controversial whether gp120 can induce signal transduction through CD4 (Horak *et al.*, 1990; Hoxie *et al.*, 1988; Kornfeld *et al.*, 1988; Mittler and Hoffmann, 1989). Cross-linking of CD4-bound gp120 by anti-gp120 MAb activated neither p56^{lck} nor Ca^{2+} mobilization (Horak *et al.*, 1990; Veillette *et al.*, 1989). However, cross-linking of CD4 by direct ligation or through CD4-bound gp120, followed by TCR cross-linking, both inhibited Ca^{2+} mobilization (Mittler and Hoffmann, 1989) and led to apoptosis (Banda *et al.*, 1992; Newell *et al.*, 1990).

CD4$^+$ T cells from asymptomatic HIV-infected individuals have also been shown to undergo apoptosis following treatment with anti-CD3, pokeweed mitogen, or staphylococcal enterotoxin B (SEB) superan-

tigen (Groux *et al.*, 1992; Meyaard *et al.*, 1992). Interestingly, gp120/gp41-mediated cell fusion that was blocked by soluble CD4 and anti-CD4 MAb (Leu3*a*) was accompanied by enhanced tyrosine phosphorylation, but not of the CD3 ζ chain (Cohen *et al.*, 1992), a known substrate of p56^{lck} (Veillette *et al.*, 1989). The CD4–gp120/gp41 interaction that occurs during cellular fusion may thus be accompanied by signal transduction involving PTK activity.

In contrast to T lymphocytes, gp120 binding to CD4 on cells of mono-cyte/macrophage lineage generally causes both activation and differentiation signals. p56^{lck} is not present in monocytes/macrophages (Bolen, 1991). Addition of soluble gp120 or inactivated HIV-1 to monocyte-derived macrophages (MDMs) had the following effects: secretion of several cytokines including IL-1β, tumor necrosis factor α (TNF-α), IL-6, and granulocyte/macrophage colony-stimulating factor (GM-CSF); secretion of arachidonic acid metabolites; and enhanced cell surface expression of MHC II antigen (Clousse *et al.*, 1991; Merrill *et al.*, 1989; Nakajima *et al.*, 1989; L. M. Wahl *et al.*, 1989; S. M. Wahl *et al.*, 1989). These effects could be blocked by soluble CD4 (Merrill *et al.*, 1989), and mimicked by anti-CD4 MAb (Merrill *et al.*, 1989; L. M. Wahl *et al.*, 1989; S. M. Wahl *et al.*, 1989). Cross-linking of CD4 with Fcγ receptors on the surface of blood monocytes or U-937 monocytes was required for the mobilization of intracellular calcium, whereas cross-linking of CD4 alone was sufficient to increase intracellular calcium levels in T lymphocytes (Guse *et al.*, 1992).

gp120 can also induce various activation signals in brain cells in a CD4-independent fashion. Addition of gp120 to rat neuron cultures led to an increase in intracellular calcium levels and cell injury (Dreyer *et al.*, 1990). binding of gp120 to a surface protein on CD4-negative glial cells induced tyrosine phosphorylation but did not lead to calcium mobilization (Schneider-Schaulies *et al.*, 1992). Finally, both soluble recombinant gp120 as well as whole HIV-1 induced the production of both IL-1 and TNF-α from rat microglia and astrocytes (Merrill *et al.*, 1992), an effect that was blocked by antibody to gp120 and gp41 but not by soluble CD4. These results suggest that gp120 may initiate signal transduction events in CD4-independent fashion, in cells derived from brain tissue.

I. Downregulation of Cell Surface CD4

Several compounds, including phorbol esters, gangliosides, 1,25-dihydroxyvitamin D$_3$, and IFN-γ, can induce cell surface depletion of CD4. This effect is also seen following MHC–antigen presentation, binding of gp120 to CD4, and differentiation of CD4$^+$CD8$^+$ thy-

mocytes to CD8$^+$ T lymphocytes. In each instance, CD4 internalization is preceded by dissociation of p56^{lck}, for example, by a CD4 cytoplasmic tail serine phosphorylation pathway, in the case of phorbol esters, or a serine phosphorylation-independent pathway, in the case of gangliosides. Alternatively, reductions in CD4 transcription and destabilization of CD4 transcripts, enhanced degradation of CD4 protein (phorbol esters), and *CD4* gene methylation (CD4$^+$CD8$^+$ thymocyte differentiation) may occur. These factors are summarized in Table III.

The phorbol ester-induced endocytosis of CD4 results from activation of PKC, which in turn phosphorylates three serine residues in the cytoplasmic tail of CD4. Phosphorylation of Ser-408 is important in inducing dissociation of CD4 and p56^{lck}, a prerequisite for CD4 internalization (Sleckman *et al.*, 1992). This effect of phorbol esters is believed to mimic the CD4–CD3 cointernalization observed in T cell activation, induced by either CD3 or CD3–CD4 cross-linking (Anderson *et al.*, 1988; Rivas *et al.*, 1988), or the more physiological situation of MHC–antigen presentation (Acres *et al.*, 1986; Rivas *et al.*, 1988; Weyand *et al.*, 1987). Cross-linking of CD4 by anti-CD4 MAb activates p56^{lck} (Veillette *et al.*, 1989) and also induces CD4–CD3 cointernalization (Cole *et al.*, 1989). Despite the fact that CD4 does not internalize with HIV-1 (Orloff *et al.*, 1991), recombinant gp120 can cointernalize in such a way as to cause dissociation of CD4 and p56^{lck} (Cefai *et al.*, 1992). CD4 internalization caused by gp120 appears to occur through a serine phosphorylation-independent pathway (Hoxie *et al.*, 1988).

Sialogangliosides induced internalization of wild-type CD4 as well as a hybrid glycolipid-anchored CD4–Thy-1 molecule that contained only the two N-terminal domains of CD4 fused to the Thy-1 antigen (Chieco-Bianchi *et al.*, 1989; Jasin *et al.*, 1991; Repke *et al.*, 1992). Thus these N-terminal domains of CD4 (D1 and D2) are sufficient to mediate ganglioside-induced CD4 endocytosis. The CD4–Thy-1 hybrid construct could not be modulated by treatment with phorbol esters because it lacked the cytoplasmic tail of CD4. Ganglioside-induced CD4 internalization was accompanied by dissociation of CD4 and p56^{lck} (Repke *et al.*, 1992), but was independent of serine phosphorylation. A minor serine phosphorylation-independent pathway of phorbol ester-induced CD4 internalization has also been reported (Shin *et al.*, 1990) and may be dependent on calmodulin and intracellular calcium, because an inhibitor of the former and an intracellular calcium chelator both blocked CD4 endocytosis (Bigby *et al.*, 1990). Thus, at least two pathways may explain dissociation of CD4 and p56^{lck} and CD4 internalization.

Transient decreases in steady state levels of CD4 mRNA following treatment with phorbol esters, in either the presence or absence of

TABLE III
MECHANISMS/FACTORS INVOLVED IN CELL SURFACE CD4 DOWNMODULATION

Mechanism/factor		HIV-1 associated	Non-HIV-1 associated	Ref.
CD4 endocytosis	Initiated by serine phosphorylation of cytoplasmic tail of CD4		Phorbol esters MHCII + Ag presentation CD3 cross-linking CD3 + CD4 co-cross-linking	Acres *et al.* (1986); Anderson *et al.* (1988); Cole *et al.* (1989); Rivas *et al.* (1988); Sleckman *et al.* (1992); Weyand *et al.* (1987)
	Serine phosphorylation independent	Cellular Nef expression (?) Soluble gp120 binding alone or with anti-gp120 antibodies	Gangliosides	Amadori *et al.* (1992); Cefai *et al.* (1992); Chieco-Bianchi *et al.* (1989); Garcia and Miller (1991); Garcia *et al.* (1993); Hoxie *et al.* (1988); Jasin *et al.* (1991); Mittler and Hoffmann (1989); Repke *et al.* (1992)
Reduced steady state levels of CD4 mRNA	Diminished or abolished CD4 transcription	Infection of CD4+ T lymphocytes or T cell lines (?)	CD4 + CD8+ thymocyte differentiation to CD8+ lymphocytes Phorbol esters Phorbol ester + anti-CD3 MAb	Geleziunas *et al.* (1991); Hoxie *et al.* (1986a); Lifson *et al.* (1986); Neudorf *et al.* (1991); Paillard *et al.* (1990); Pimentel-Muinos *et al.* (1992); Richardson *et al.* (1986); Salmon *et al.* (1988); Stevenson *et al.* (1987); von Boehmer (1988)

(continued)

TABLE III   (*Continued*)

Mechanism/factor	HIV-1 associated	Non-HIV-1 associated	Ref.
CD4 mRNA degradation		Phorbol ester + anti-CD3 MAb	Paillard *et al.* (1990)
Diminished CD4 translation	Infection of T lympho-cyte and monocytic cell lines		Geleziunas *et al.* (1991); Hoxie *et al.* (1986a); Yuille *et al.* (1988)
Intracellular reten-tion by inter-molecular complex formation	CD4–gp160 heterodimer blocked in ER		Bour *et al.* (1991); Crise *et al.* (1990); Crise and Rose (1992)
CD4 degradation	Caused by Vpu and occurring in the ER	Following phorbol ester-induced endocytosis and occurring in lysosomes	Munck Peterson *et al.* (1992); Shin *et al.* (1991b); Willey *et al.* (1992a,b)
CD4 shedding	CD4 association with budding virions		Meerloo *et al.* (1992)
Unknown	Treatment of monocytes with either 1,25-dihydroxyvitamin D_3 or IFN-γ		Faltynek *et al.* (1989); Rigby *et al.* (1990)

anti-CD3 MAb, have been attributed to a highly diminished rate of CD4 transcription (Neudorf *et al.*, 1991; Paillard *et al.*, 1990; Pimentel-Muinos *et al.*, 1992) and destabilization of CD4 transcripts (Paillard *et al.*, 1990). Exposure of cells that expressed CD4 under control of a heterologous promoter to phorbol esters also led to CD4 internalization; however, reestablishment of cell surface CD4 occurred both more rapidly and to a greater extent in comparison with cells that expressed wild-type CD4 (Neudorf *et al.*, 1991). Thus, reductions in CD4 transcription following treatment with phorbol esters may delay the reestablishment of cell surface CD4.

A T cell-specific transcriptional enhancer located approximately 13 kb upstream of the transcription initiation site for murine CD4 has been identified (Sawada and Littman, 1991). This element contains three nuclear protein-binding sites, one of which binds the T cell-specific transcription factor TCF-1α/LEF-1, whereas the other two apparently bind basic helix–loop–helix proteins. Interactions between these transcriptional factors may be important for optimal CD4 enhancer activity.

Endocytosis of CD4 following treatment with phorbol ester led to accumulation of this molecule within lysosomes, where it was degraded (Munck Peterson *et al.*, 1992; Shin *et al.*, 1991b). Treatment of monocytic cell lines with IFN-γ and monocyte-derived macrophages (MDMs) with either IFN-γ or 1,25-dihydroxyvitamin D_3 also caused reductions in cell surface CD4 (Faltynek *et al.*, 1989; Rigby *et al.*, 1990). The IFN-γ-induced reduction was cell lineage dependent, because similar treatment of peripheral blood T lymphocytes or the MOLT-4 T cell line did not result in CD4 modulation (Faltynek *et al.*, 1989). Both compounds had lesser effects on CD4 modulation in monocytes than phorbol esters (Faltynek *et al.*, 1989; Munck Peterson *et al.*, 1992).

The *CD4* gene is specifically downregulated in CD4+CD8+ (double-positive) thymocytes, which possess a MHC I-specific TCR, during the process of positive selection. Specifically, thymocytes that bear TCRs that recognize MHC I antigens retain CD8 but downmodulate expression of CD4 (von Boehmer, 1988). A similar reciprocal pattern of negative regulation apparently associated with gene methylation involves the *CD8* gene in double-positive, MHC II-specific thymocytes. Progressive demethylation of the *CD8* gene has been observed during the transition from CD4−CD8− (double-negative) to double-positive thymocytes (Carbone *et al.*, 1988a). Fusion of murine CD8+ T lymphocytes, in which the *CD8* gene was undermethylated, with a CD8− thymoma, in which the *CD8* gene was heavily methylated, generated hybridomas that did not express CD8 (Carbone *et al.*, 1988b). The loss

of CD8 expression was attributed to transcriptional inhibition and appeared to correlate with remethylation of the *CD8* gene (Carbone *et al.*, 1988b).

These observations are consistent with the finding that treatment of CD8⁺ T cells with the DNA methylation antagonist 5-azacytidine induced CD4 expression (Richardson *et al.*, 1986). Infection of CD8⁺ T cells by human herpesvirus-6 (HHV-6) also induced CD4 expression, as documented both at the transcriptional level and in terms of HIV-1 receptor activity (Lusso *et al.*, 1991).

IV. CD4 DOWNREGULATION BY HIV-1: MECHANISMS AND VIRAL GENES

A. *HIV-1 Virion Structure, Genomic Organization, and Temporal Gene Expression*

HIV-1 is a lentivirus that shares with certain other retroviruses [including all lentiviruses, spumaviruses, and human T cell leukemia viruses (HTLV)] such characteristics as a complex genome and a temporal pattern of gene expression, attributable to the action of two viral regulatory proteins termed Tat and Rev (Cullen, 1991a). Simple retroviruses such as murine leukemia viruses (MLVs) and avian leukemia viruses (ALV) lack these attributes. As depicted in Fig. 2, HIV-1 possesses three genes that characterize all replication-competent retroviruses; these are arranged in the order 5′ *gag–pol–env* 3′ and are flanked by long terminal repeats (LTRs). Both LTRs are identical, but the 5′ LTR contains transcriptional regulatory sequences whereas the 3′ LTR is functionally important for mRNA polyadenylation. HIV-1 also possesses six regulatory genes: *tat, rev, vif, vpr, vpu,* and *nef* (Cullen, 1991b).

The *gag* gene encodes viral structural proteins of which p17 and p24 comprise the matrix (MA) and capsid (CA), respectively, whereas p7/p9 binds to viral RNA and make up the viral nucleocapsid (NC) (Fig. 2). The *pol* gene encodes three distinct enzymes: protease (p11PR), reverse transcriptase (p66/p51RT), and integrase (p32IN). The protease exists as a homodimer and processes the p160$^{gag–pol}$ precursor protein into mature products. The reverse transcriptase (RT) is a heterodimer of 66 and 51 kDa; the latter subunit lacks the C-terminal end associated with the former and that possesses RNase H activity. The RT possesses both RNA- and DNA-dependent DNA polymerase activity and catalyzes the synthesis of proviral DNA from viral genomic RNA. The viral integrase is responsible for integration of double-stranded viral DNA into host cell DNA. Finally, the *env* gene encodes a precursor termed gp160 that oligomerizes prior to cleavage, generating the ma-
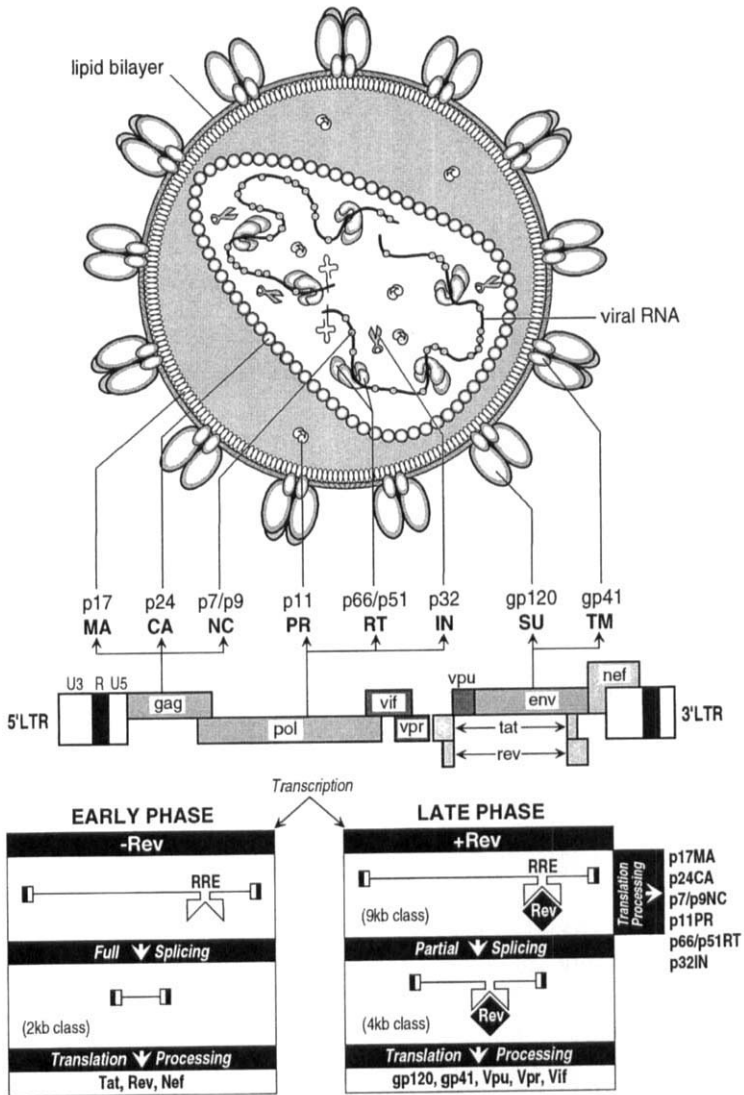
FIG. 2.   HIV-1 virion structure, genomic organization, and temporal gene expression.

ture envelope glycoproteins gp120 (SU) and gp41 (TM), which are present as either dimers or tetramers at the surface of the virion (Fig. 2) (reviewed in Camerini and Chen, 1991; Capon and Ward, 1991; Greene, 1990, 1991).

Both Tat and Rev have been extensively studied and shown to be essential for HIV-1 replication (Cullen, 1991a). These are both viral

RNA-binding proteins and are encoded by genes that are divided into two coding exons (Fig. 2). Tat binds to a specific RNA stem–loop structure termed the TAR (*trans*-activation responsive element) located at the beginning of the R segment of the 5′ LTR. Binding of Tat to the TAR dramatically increases HIV-1 LTR-driven gene expression, leading to high levels of viral mRNA and proteins (Cullen, 1991a). Rev binds to a highly structured RNA segment termed the RRE (Rev responsive element) located within the *env* gene.

HIV-1 proteins are expressed in a temporally regulated fashion. The regulatory proteins Tat, Rev, and Nef are expressed during the early phase of viral gene expression whereas the *gag, pol, env, vpu, vpr*, and *vif* gene products are expressed later (Cullen, 1991a,b) (Fig. 2, bottom). This is due to the synthesis of the former proteins from fully spliced mRNAs (2-kb class), which are independent of Rev, whereas the latter proteins are generated from partially spliced (4-kb class) or unspliced (9-kb class) mRNAs that require a threshold level of Rev protein for expression (Fig. 2, bottom). Thus, the switch from the early regulatory phase of HIV-1 gene expression to the late structural phase is controlled by levels of Rev protein (Cullen, 1991a).

The function of Nef (negative factor) is controversial. Initially, this protein was shown to repress HIV-1 LTR-driven transcription, and deletion of the *nef* gene was shown to enhance viral replication. However, subsequent reports did not corroborate these findings (reviewed in Cullen, 1991b; Greene, 1990, 1991). Nef has also been shown to downmodulate cell surface CD4 expression (Garcia and Miller, 1991; Garcia *et al.*, 1993; Guy *et al.*, 1987). Vpu has been found only in HIV-1 and can enhance virion release from infected cells, possibly by inhibiting the budding of viral particles through intracytoplasmic membranes (Klimkait *et al.*, 1990; Terwilliger *et al.*, 1989). Vpu can also reduce CD4 levels (Willey *et al.*, 1992a,b). Vif is important for infectivity, because virions produced from proviruses lacking *vif* are less infectious than wild-type virions. Vpr is virion associated and may enhance viral replication (reviewed in Cullen and Greene, 1990).

This chapter focuses on the role of gp160, gp120, Vpu, and Nef in downmodulation of CD4. Both Vpu and gp160 are generated from the same bicistronic mRNA (4-kb class) during the late phase of viral replication and are dependent on Rev, whereas Nef is made during the early regulatory phase of HIV-1 gene expression (see Fig. 2, bottom).

B. Factors Involved in HIV-1-Associated CD4 Downmodulation

HIV infection in culture with CD4$^+$ lymphocytes, monocyte-derived macrophages, T cell lines, and monocytic cell lines leads to cell surface

CD4 downmodulation (Folks *et al.*, 1985; Geleziunas *et al.*, 1991; Hoxie *et al.*, 1985, 1986a; Melendez-Guerrero *et al.*, 1990; Salmon *et al.*, 1988; Stevenson *et al.*, 1987; Yuille *et al.*, 1988). First, the masking of CD4 may occur early in infection as a consequence of the binding of virions, soluble gp120, or gp120–anti-gp120 antibodies to the cell surface (Amadori *et al.*, 1992; Hart and Cloyd, 1990; McDougal *et al.*, 1986). In addition, binding to CD4 of soluble gp120 or gp120–anti-gp120 antibodies may cause CD4 internalization (Amadori *et al.*, 1992; Cefai *et al.*, 1992; Mittler and Hoffmann, 1989). Nef expression, which precedes synthesis of HIV-1 structural proteins, may also cause cell surface CD4 downregulation (Garcia and Miller, 1991).

During early HIV-1 viral production, CD4, along with other host proteins, was shown to associate with budding virions, representing another relatively early factor in cell surface CD4 depletion (Meerloo *et al.*, 1992). At a later stage in the viral life cycle, CD4 expression may be affected at three different levels. First, CD4 transcript levels are reduced both in infected T cell lines and normal CD4$^+$ lymphocytes (Geleziunas *et al.*, 1991; Hoxie *et al.*, 1986a; Salmon *et al.*, 1988). Although mechanisms to explain this observation are ill defined, possible explanations include the outgrowth of low CD4-expressing variants that are resistant to HIV-1-induced cytopathicity (Lifson *et al.*, 1986). Reduced levels of immunoprecipitable CD4 have also been observed (Hoxie *et al.*, 1986a) and may be due to impaired translation of CD4 mRNA (Geleziunas *et al.*, 1991; Yuille *et al.*, 1988) or Vpu-associated degradation of CD4 (Willey *et al.*, 1992a,b). Finally, the formation of CD4–gp160 complexes may represent a further event in this process (Bour *et al.*, 1991), because cell lines that expressed CD4 and the *env* gene possessed such complexes and had reduced levels of cell surface CD4 (Crise *et al.*, 1990; Jabbar and Nayak, 1990; Kawamura *et al.*, 1989; Stevenson *et al.*, 1988) (Table III). One consequence of cell surface CD4 depletion, by either gp120-induced internalization or HIV-1 infection, is refractoriness to T cell activation mediated by the CD3–TCR complex (Cefai *et al.*, 1992; Linette *et al.*, 1988). This is most likely due to the absence of CD4, which serves as an important coreceptor during this activation process (Janeway, 1992).

C. Maturation of gp160

The *env* gene encodes the gp160 precursor protein that is cleaved into the surface (SU) gp120 and transmembrane (TM) gp41 subunits. gp120 is responsible for CD4 recognition and is noncovalently associated with gp41, which possesses fusion activity and anchors gp120 into

the viral envelope or plasma membrane (Fig. 2). This noncovalent linkage permits extensive shedding of gp120 (Schneider *et al.*, 1986).

gp160 is synthesized from a bicistronic mRNA that contains *vpu* coding sequences at its 5' end (Schwartz *et al.*, 1990). Synthesis of gp160 occurs when the *vpu* AUG (initiation codon) is bypassed during ribosomal scanning (leaky scanning) of the bicistronic *vpu–env* mRNA molecules (Schwartz *et al.*, 1992). The *vpu–env* mRNA is dependent on Rev for expression (Fig. 2, bottom). Rev functions by activating the transport of RRE-containing transcripts, such as *vpu–env*, from the nucleus to the cytoplasm. Rev, by binding to RRE-containing mRNA, may antagonize the interaction of splicing factors with such transcripts or may facilitate their interaction with components involved in nuclear export (Cullen, 1991a) (Fig. 2, bottom). In addition, Rev may be required for the translation of *vpu–env* mRNAs (Arrigo and Chen, 1991).

The presence of a leader sequence containing a hydrophobic signal peptide at the N terminus of gp160 (Ellerbrok *et al.*, 1992) directs the nascent protein through the secretory pathway, that is, the ER and the Golgi apparatus (Earl *et al.*, 1991; Willey *et al.*, 1991). The leader sequence is removed by the signal peptidase (Fig. 3A) in the ER during translation, and the translocation process is stopped by a hydrophobic sequence of amino acids corresponding to the membrane anchorage region of gp41 (Fig. 3A) located at the C terminus (Hunter and Swanstrom, 1991).

Early events of gp160 maturation occur in the ER. These include disulfide bond formation, folding into a conformation competent to bind CD4, addition of high-mannose oligosaccharide side chains through N-linked glycosylation of asparagine residues, and gp160 dimerization (Earl *et al.*, 1991; Fennie and Lasky, 1989; Haseltine, 1991). The initially generated high-mannose structures of gp160 contain three glucose residues, which are removed by α-glucosidases I and II in the ER. Further trimming and addition of carbohydrates occurs in the Golgi complex (Elbein, 1991). Inhibitors of α-glucosidase activity have been shown to be effective antagonists of production of infectious HIV-1 (see below).

Early events affecting gp160 in the ER occur in the following sequence (Earl *et al.*, 1991): disulfide bond formation ($t_{1/2} \approx 10$ min), acquisition of CD4-binding competency ($t_{1/2} \approx 15$ min), and transient association with the heavy chain-binding protein (BiP, GRP78) ($t_{1/2} \approx 25$ min), which is believed to promote folding and oligomerization. In mammalian cells that expressed a secreted form of gp120, proper folding and CD4 recognition occurred with a half-life of approximately 30 min (Fennie and Lasky, 1989). Finally, dimerization of gp160 also takes place ($t_{1/2} \approx 30$ min) in the ER. Acquisition of CD4-binding
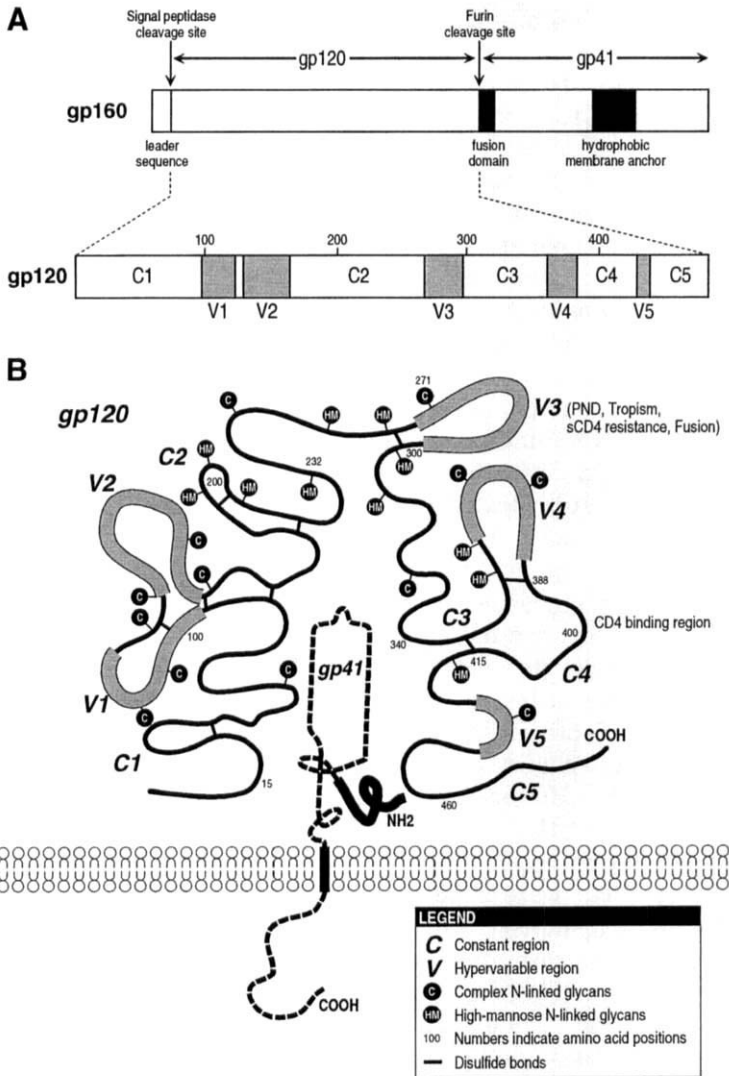
FIG. 3. HIV-1 envelope glycoproteins. (A) Linear representations of gp160 and gp120. (B) Hypothetical structure of gp120 and gp41 at cell or virion surface. [After Gallaher *et al.* (1989) and Leonard *et al.* (1990).] Amino acid numbering is according to Leonard *et al.* (1990) and begins with the first threonine residue of mature gp120.

ability and dimerization were not affected by inhibitors of transport from the ER to the Golgi, proving that both events occur in the former compartment (Earl *et al.*, 1991; Fennie and Lasky, 1989).

Cleavage of gp160 and acquisition of complex carbohydrate structures occur in the Golgi (Stein and Engleman, 1990) with an approxi-

mate half-life of 80 min (Earl *et al.*, 1991). Approximately 50% of the gp120 cleavage product is shed by about 120 min (Earl *et al.*, 1991). The lag between gp160 dimerization ($t_{1/2} \approx 30$ min) and gp160 cleavage ($t_{1/2} \approx 80$ min) thus represents a rate-limiting step of gp160 maturation and shedding of gp120.

All 18 conserved cysteines of a recombinant gp120 molecule were shown to participate in 9 intrachain disulfide bonds forming 5 distinct loop structures (Leonard *et al.*, 1990) important in tertiary structure (Fig. 3B). The first and fourth loops (C1 and V3) have single bonds whereas the loops formed by VIV2, C2, and V4C4 are maintained by nested disulfide bridges (Leonard *et al.*, 1990) (Fig. 3B). When cysteines at positions 266 or 301, in V3, were changed to valines, the resulting virus was noninfectious due to poor gp160 cleavage and impairment of CD4 binding (Tschachler *et al.*, 1990). Alterations of Cys-388 in V4 or Cys-415 in C4 likewise reduced levels of binding to CD4. However, substitution of Cys-355, which pairs with Cys-388 to form a disulfide bond in V4, was less detrimental to infectiousness. Finally, substitution of Cys-101 in V1 or Cys-166 involved in both V1 and V2 (Fig. 3B) yielded viruses that were both noninfectious and defective in syncytium-inducing ability (Tschachler *et al.*, 1990).

The gp120 molecule is heavily glycosylated, with approximately half of its mass being carbohydrate. All 24 asparagine residues of a recombinant gp120, representing consensus sites for N-glycosylation (Asn-X-Ser/Thr), were shown to be linked to oligosaccharides (Leonard *et al.*, 1990) (Fig. 3B). However, only half of these sites were conserved among sequenced HIV-1 isolates (Myers, 1990). When gp160 dimers transit the Golgi apparatus, 13 of 24 high-mannose core oligosaccharides in gp120 were found to be further modified by mannosidases and glycosyltransferases, which trim mannose residues and add additional *N*-acetylglucosamine, galactose, fucose, and sialic acid residues (Ellerbrok *et al.*, 1992; Leonard *et al.*, 1990; Ratner *et al.*, 1991) (Fig. 3B). The oligosaccharide side chains of gp160, gp120, and gp41 were also shown to be sulfated (Bernstein and Compans, 1992) by sulfotransferases in the *trans*-Golgi, contributing to overall negative charge.

The asparagine residues of the N-linked glycosylation sites of gp120 are less critical for viral infectivity than the cysteines in disulfide bonds. When biologically active molecular clones of HIV-1 (HXB2) containing mutations in each of the asparagine residues of gp120 were examined for infectiousness, only five such mutants (positions 58, 111, 167, 232, and 246) showed delayed replication kinetics (Lee *et al.*, 1992a). In another study on the NL4-3 molecular clone of HIV-1, asparagine mutations at positions 211, 246, and 259 had no effect on viral

replication, whereas alteration of Asp-232 caused noninfectiousness in spite of the ability to bind CD4 (Willey *et al.*, 1988b). In the latter study, mutations at adjacent residues possessed similar phenotypes, suggesting that loss of the N-glycosylation site was not responsible for loss of infectivity. Thus, most N-linked glycosylation sites on gp120 are dispensable for infectivity (Gabuzda *et al.*, 1992). Consistent with these results is that enzymatically deglycosylated gp120 is capable of recognizing CD4, albeit with reduced affinity (Fenouillet *et al.*, 1989). However, deglycosylation of gp120 in the presence of detergent caused a 50-fold lower affinity for CD4 (Matthews *et al.*, 1987), suggesting that carbohydrates may be required to maintain the gp120 conformation necessary for CD4 binding (Fennie and Lasky, 1989).

Among a large number of aminosugar derivatives, *N*-butyldeoxynojirimicin, an inhibitor of ER-associated α-glucosidase I, was shown to have potent anti-HIV-1 activity (Karpas *et al.*, 1988). Treatment of infected cells with either this compound or castanospermine, another α-glucosidase I inhibitor, blocked syncytium formation and yielded particles of diminished infectivity (Gruters *et al.*, 1987; Montefiori *et al.*, 1988; Pal *et al.*, 1989; Ratner *et al.*, 1991; Walker *et al.*, 1987). This inhibitory effect was attributed, in part, to reduced and delayed processing of gp160 into gp120 and gp41, probably due to incomplete oligosaccharide processing (Montefiori *et al.*, 1988; Pal *et al.*, 1989; Ratner *et al.*, 1991; Walker *et al.*, 1987). Both gp160 and gp120 from drug-treated cells had higher than usual molecular weights, suggesting incomplete trimming of oligosaccharides. However, gp120 from such cells maintained ability to bind CD4. In contrast, inhibitors of the Golgi-associated mannosidases I and II did not possess anti-HIV-1 properties (Gruters *et al.*, 1987; Pal *et al.*, 1989). Thus, N-linked oligosaccharides are most likely important for both proper folding of viral glycoproteins and their conformational stabilization (Paulson, 1989). Carbohydrates on HIV-1 envelope glycoproteins may also reduce their immunogenicity by masking polypeptide epitopes.

Viral gp160 dimerizes prior to its exit from the ER (Earl *et al.*, 1991). Proper folding and oligomerization of retroviral envelope precursors were shown to be a prerequisite for transport to the Golgi complex (Hunter and Swanstrom, 1991). however, oligomerization is not sufficient to ensure transport of viral envelope glycoproteins to the Golgi. A HIV-1 proviral clone carrying a single amino acid substitution in the C2 domain of gp120 (Fig. 3) was reported to form gp160 oligomers that were blocked in the ER (Willey *et al.*, 1991), possibly representing improper folding.

Homodimers of gp160 are associated noncovalently and may further lead to formation of tetramers (Earl *et al.*, 1990, 1991; Schawaller *et*

al., 1989). The oligomeric glycoprotein structures are cleaved into gp120 and gp41 subunits in the Golgi (Stein and Engleman, 1990) and are subsequently expressed at both the cell and viral surface as dimers or tetramers (Schawaller et al., 1989; Weiss et al., 1990) (Fig. 2). These oligomeric structures are capable of multimeric association with CD4, which may increase the avidity of HIV binding (Earl et al., 1990). The N-terminal ectodomain of gp41, which contains the fusion peptide of this molecule, also represents the oligomer assembly region (Earl et al., 1990; Pinter et al., 1989) (Fig. 3). HIV-1 gp160 can form hetero-dimers with envelope precursors of both SIV and HIV-2, indicating that all three primate lentiviruses possess functionally conserved en-velope assembly domains (Doms et al., 1990).

Proteolytic cleavage of gp160 into gp120 and gp41 exposes the N-ter-minal fusion domain of the latter protein, which is essential for HIV-1 infectivity and membrane fusion (Bosch and Pawlita, 1990; McCune et al., 1988; O'Hara et al., 1990; Willey et al., 1989). The last four C-ter-minal amino acids of gp120 (Arg-Glu-Lys-Arg) correspond to a con-served endoproteolytic cleavage site (Arg-X-Lys/Arg-Arg) found in many retroviral envelopes (McCune et al., 1988). Cleavage occurs after the last arginine residue. Replacement of this entire sequence, or sub-stitution of the last arginine residue by serine or threonine (but not lysine), abolished gp160 processing and syncytium formation but did not affect transport and cell surface expression of gp160 (Bosch and Pawlita, 1990; Freed et al., 1992; Guo et al., 1990; McCune et al., 1988). Other mutations that affected an alternative upstream cleavage site (Bosch and Pawlita, 1990) or residues in both cleavage sites (Willey et al., 1991) had similar effects on viral infectivity.

The host enzyme that cleaves gp160 is furin, a subtilisin-like eu-karyotic endoprotease found in the Golgi complex (Hallenberger et al., 1992). Furin is also responsible for cleavage of an avian influenza virus hemagglutinin that bears the same consensus proteolytic site as gp160 (Hallenberger et al., 1992). Although less than 25% of gp160 is cleaved into gp120 and gp41 in T lymphocytes, with the remainder undergoing lysosomal degradation (Earl et al., 1991; Willey et al., 1988a), the effi-ciency of this cleavage may vary among cell types (Earl et al., 1991).

D. Functional Domains of gp120 and gp41

Important functions and regions of gp120 include CD4 binding (C4), macrophage tropism, sensitivity to neutralization by soluble CD4, fu-sion (V3), infectivity (C2), and association with gp41 (C1, C3, C4, C5) (Fig. 3B). Important regions of gp41 include the hydrophobic trans-

membrane anchor and amino-terminal fusion domain (Fig. 3A), as well as the dimerization and gp120 association regions.

1. gp120

Comparison of several HIV-1 gp120 sequences revealed five hypervariable regions (V1–5) interspersed with conserved regions (C1–5) (Fig. 3). Computer-generated models suggest that the hypervariable regions represent potential antigenic sites (Modrow *et al.*, 1987; Willey *et al.*, 1986).

Site-specific mutagenesis that altered amino acids Leu-231, Asn-232, or Gly-233 of the C2 domain of gp120 eliminated infectiousness but not binding to CD4 (Willey *et al.*, 1988b). Spontaneous tissue culture revertants had a mutation changing Ser-98 to Asn in V1. Mutagenesis of the Ser-98 codon, followed by *in vitro* passage, generated a third revertant involving a change in V3 (Arg-274→Ile) (Willey *et al.*, 1989). Thus, following binding to CD4 all three regions (C2, V1, and V3) may lie in proximity or interact with one other; all three are apparently involved in mediating HIV-1 infectivity subsequent to receptor binding.

Epitope mapping involving gp120-specific MAbs and mutagenesis studies have shown that a C4 region that includes amino acids 389–407 is essential in CD4 binding (Cordonnier *et al.*, 1989; Kowalski *et al.*, 1987; Lasky *et al.*, 1987; Olshevsky *et al.*, 1990). Substitution of Trp-397 also impaired association between gp120 and gp41, suggesting that alterations in tertiary structure had occurred and implicating (Olshevsky *et al.*, 1990) this residue in CD4 binding through preservation of proper conformation. Alteration of three other residues in C4, that is, Ala-403 (Lasky *et al.*, 1987), Ile-390 (Cordonnier *et al.*, 1989), and Asp-427 (Olshevsky *et al.*, 1990), also diminished CD4 binding, although altering Asp-427 did not destroy gp120 conformation (Olshevsky *et al.*, 1990).

Mutagenesis outside of C4 did not significantly disrupt gp120 tertiary structure but decreased CD4-binding ability. Alterations of Asp-338 and Glu-340 in C3 led to greater than 100-fold reductions in CD4 binding whereas changing Thr-227 in the C2 region had less dramatic consequences (Olshevsky *et al.*, 1990). Thus, residues proximal to and including Trp-397 may be important in preserving a conformation able to bind CD4 whereas residues Thr-227 (C2), Asp-338 (C3), Glu-340 (C3), and Asp-427 (C4) may be proximal (on a properly folded gp120 molecule) and directly contact CD4. Human monoclonal antibodies that blocked gp120–CD4 binding reacted against four discontinuous regions of gp120 that overlapped the discontinuous residues involved

in CD4 binding (Thali *et al.*, 1992). Consistent with these epitope mapping and mutagenesis studies is the observation that a truncated gp120 molecule could still bind CD4, in spite of deletions of 62 N-terminal residues of C1, 20 C-terminal residues of C5, and deletions of the V1, V2, and V3 domains (Pollard *et al.*, 1992).

The principal neutralizing determinant (PND) of HIV-1 is located in the V3 loop of gp120, deletion of which renders gp120 unable to elicit neutralizing antibodies (Javaherian *et al.*, 1989). The neutralizing determinant is located at the tip of the loop and contains a conserved Gly_{282}-Pro_{283}-Gly_{284} motif that is flanked by highly variable residues. Antibodies against V3 block infectivity and inhibit cell fusion but do not prevent gp120 binding to CD4 (Javaherian *et al.*, 1989; Skinner *et al.*, 1988). Mutations that altered either the Gly-Pro-Gly sequence or certain adjacent residues, including Arg-285 (depending on viral isolate), abolished or greatly reduced syncytium formation and infectiousness (Freed *et al.*, 1990; Grimaila *et al.*, 1992; Page *et al.*, 1992). However, such mutants were not impaired in either synthesis, processing, or transport of gp160, or in its ability to bind CD4, demonstrating the involvement of V3 in fusion.

Several reports suggest that the tip of V3 may represent a cleavage site for trypsin-like proteases. First, sequence homology exists between this site and a peptide inhibitor of trypsin-like proteases that block HIV-1-associated syncytium formation (Hattori *et al.*, 1989). Second, the V3 loop can be cleaved by trypsin-like enzymes (Clements *et al.*, 1991). Third, recombinant gp120 can sometimes be naturally cleaved at the tip of V3 (Stephen *et al.*, 1990). Thus, HIV-1 attachment to the CD4 receptor may be followed by cleavage of the V3 loop mediated by membrane-associated proteases, leading to alterations in gp120 conformation and exposure of the N-terminal fusion domain of gp41.

Studies have suggested a structural relationship between the V3 loop and the C4 domain. Lysates of cells (prepared with ionic detergents) that expressed wild-type or V3-deleted HIV glycoproteins were treated with MAbs reactive with the discontinuous epitopes involved in CD4 binding. These MAbs precipitated wild-type glycoproteins less efficiently than did V3 loop mutants (Wyatt *et al.*, 1992), possibly due to V3 loop masking of the CD4-binding region. Moreover, amino acid changes at the base of the V3 loop (Arg-268) or in C4 (Trp-397) resulted in increased exposure of epitopes that overlap the CD4-binding region (Wyatt *et al.*, 1992).

The V3 loop has also been shown to be the major determinant of macrophage and T cell line tropism and susceptibility to neutralization by soluble CD4 (sCD4) (Hwang *et al.*, 1991, 1992; O'Brien *et al.*, 1992).

Replacement of V3 loop sequences of T cell tropic isolates with sequences of macrophage-tropic isolates conferred ability to grow in macrophages (Hwang *et al.*, 1991; O'Brien *et al.*, 1990; Shioda *et al.*, 1991). Hybrid viruses bearing only macrophage-tropic V3 loop sequences were more resistant than the original T cell-tropic viruses to sCD4 neutralization, a resistance shared with primary macrophage-tropic HIV-1 isolates (Hwang *et al.*, 1992; O'Brien *et al.*, 1992). V3 loop sequences of macrophage-tropic HIV-1 types, which predominate early in disease, resemble each other and represent a consensus sequence, unlike the divergent V3 loops of T cell line-tropic viruses that become more prevalent as disease progresses (Hwang *et al.*, 1991).

Amino acid insertions at the N termini of both gp120 and gp41 disrupted association of these molecules (Kowalski *et al.*, 1987) (Fig. 3). Substitution mutations in the highly conserved N-terminal C1 domain, encompassing residues 6 to 15 of gp120, or deletion of the N-terminal 31 amino acids of gp120 disrupted noncovalent gp120 and gp41 association (Helseth *et al.*, 1991; Ivey-Hoyle *et al.*, 1991). Additional residues at positions 461–471 located in the conserved C5 domain of the C-terminal end of gp120 are also important for gp41 association. Less dramatic dissociation of gp120 and gp41 was observed following mutagenesis that altered residues in both C3 (residues 350–354) and C4 (residues 390–397 and 403–408) (Helseth *et al.*, 1991). Mutations and deletions in these regions did not significantly affect gp160 processing or CD4 binding, suggesting that overall conformation of gp120 was not disrupted. Other mutations in gp120 that caused disruption of gp120–gp41 association also decreased gp160 processing and CD4 binding (Helseth *et al.*, 1991).

2. gp41

Of two hydrophobic regions in gp41, one represents a transmembrane anchor that causes the molecule to span the lipid bilayer while the other, at the N terminus (Fig. 3A), shares sequence homology with the fusion peptides of ortho- and paramyxoviruses (Bosch *et al.*, 1989; Gallaher, 1987). Linker insertion mutations in the N-terminal HIV-1 fusion domain or replacement of apolar with polar amino acids reduced or abolished syncytium formation (Bosch *et al.*, 1989; Freed *et al.*, 1989; Kowalski *et al.*, 1987). One such mutation (Val-2→Glu), resulted in an envelope that dominantly interfered with wild-type envelope in this respect (Freed *et al.*, 1990). In SIV, mutations that increased the overall hydrophobicity of the equivalent gp32 N terminus enhanced syncytium formation (Bosch *et al.*, 1989). Introduction of mutations into the fusogenic domain of SIV gp32 abolished syncytium formation, perhaps by interfering with fusion peptide insertion into

lipid bilayers (Horth *et al.*, 1991). Thus, the hydrophobic N terminus of HIV-1 gp41 (Fig. 3A) and its equivalent region in SIV gp32 are involved in membrane fusion.

The putative structure of gp41 was predicted on the basis of TM protein properties and comparisons with the structure of the influenza virus HA_2 transmembrane protein (Gallaher *et al.*, 1989) (Fig. 3B). Two features distinguish gp41 from other retroviral TM properties. First, its membrane-spanning segment, like that of certain other lentiviruses, is punctuated by one or two polar residues; in the case of most other retroviruses, this region consists of uninterrupted apolar amino acids (Hunter and Swanstrom, 1991). Alteration of the two charged residues (Lys and Arg) in this segment abolished both syncytium formation and viral replication, without affecting other envelope properties such as binding to CD4 (Helseth *et al.*, 1990).

Second, the cytoplasmic domains of retroviral TM properties are usually short (22–38 residues); however, that of HIV-1 is relatively long (150 residues) (Hunter and Swanstrom, 1991). Introduction of deletions or termination codons in the cytoplasmic domain of gp41 showed that this region was important for infectiousness (Dubay *et al.*, 1992b; Freed *et al.*, 1991), although not for envelope glycoprotein synthesis or processing, CD4 binding, or syncytium formation. Thus, the cytoplasmic domain of gp41 is crucial for HIV-1 entry although not for receptor binding or membrane fusion. Loss of infectiousness may be related to reduced incorporation of truncated gp41 mutants into virions (Dubay *et al.*, 1992b). Vif, an auxiliary protein important in infectivity, possesses cysteine protease activity that can modify the cytoplasmic domain of gp41 (Guy *et al.*, 1991).

Other mutational studies showed that elimination of N-linked glycosylation sites in the gp41 ectodomain reduced viral infectivity (Dedera *et al.*, 1992b; Lee *et al.*, 1992b). The gp41 ectodomain contains a leucine zipper-like motif that is located downstream of the fusion peptide. This motif is also found in other retroviral TM proteins and may be important in envelope oligomerization. Replacement of a central isoleucine residue of this motif by nonconservative amino acids inhibited fusion and infectivity without affecting envelope synthesis, oligomer formation, transport, and processing (Dubay *et al.*, 1992a). These results demonstrate the importance of the gp41 leucine zipper motif in membrane fusion. The C-terminal half of this motif represents an immunosuppressive sequence (residues 581–597) homologous to that found in TM proteins of other retroviruses (Ruegg and Strand, 1990). When internalized, this peptide inhibits lymphoproliferation by antagonizing PKC activity (Ruegg and Strand, 1990). Two closely spaced cysteine residues immediately downstream of the leucine zip-

per motif are highly conserved among animal retroviruses; mutation of either residue caused loss of viral infectivity attributable to impaired gp160 processing (Dedera et al., 1992a; Syu et al., 1991).

HIV-1 infection of cells lacking LFA-1, a leukocyte adhesion molecule, was not accompanied by syncytium formation (Pantaleo et al., 1991). Antibodies against the β subunit (CD18) of LFA-1 were shown to inhibit virus-induced syncytium formation (Valentin et al., 1990), indicating that both LFA-1 and gp41 may be required for cell fusion, and that interactions may be likely to occur between these two molecules.

E. CD4–gp160 Complexes

Productive HIV-1 infection leads to cell surface depletion of CD4 (Geleziunas et al., 1991; Hoxie et al., 1986a; Salmon et al., 1988; Stevenson et al., 1987; Yuille et al., 1988), rendering cells refractory to superinfection (Hart and Cloyd, 1990). First, treatment of chronically infected promyelocytic cells with tumor necrosis factor α (TNF-α) led to a dramatic increase in HIV-1 expression concomitant with a rapid decrease of expression of cell surface CD4 (Butera et al., 1991). Surface CD4 levels returned to normal when viral expression dropped, following TNF-α activation, but could again by downmodulated following HIV reactivation. Second, treatment of a chronically HIV-1-infected T cell line that lacked cell surface CD4 with a Tat antagonist caused inhibition of viral replication and increased cell surface CD4 representation (Shahabuddin et al., 1992). Finally, acute infection of a T cell line caused a progressive decline of surface CD4 over 9 days, concomitant with increasing resistance to infection by HIV-2 (Hart and Cloyd, 1990). In each case, levels of cell surface CD4 were inversely proportional to abundance of gp160.

In productively infected cells, CD4 is associated intracellularly with gp160 and sometimes with gp120 (Bour et al., 1991; Geleziunas et al., 1991; Hoxie et al., 1986a; Salmon et al., 1988; Shahabuddin et al., 1992; Yuille et al., 1988). Formation of CD4–gp160 complexes sequesters CD4 within the cell. The role of such complexes in cell surface CD4 depletion was demonstrated in both lymphocytic and monocytic cells that were engineered to express gp160; diminished levels of surface CD4 were inversely correlated with levels of gp160 (Kawamura et al., 1989; Stevenson et al., 1988). Expression of gp160 in CD4$^+$ cells but not in CD4$^-$ cells resulted in cytopathic effects, leading to death in the absence of syncytium formation (Koga et al., 1990b). These complexes also include the p56^{lck} PTK, by virtue of its association with CD4; the complexes are formed and retained in the ER, thus inhibiting matura-

tion and transport of both CD4 and gp160 (Bour *et al.*, 1991; Crise *et al.*, 1990; Crise and Rose, 1992; Jabbar and Nayak, 1990). In contrast, complexes between CD4 and gp120 can be efficiently transported from the ER (Crise *et al.*, 1990), suggesting that membrane anchorage of gp160, mediated by the C-terminal transmembrane segment in gp41, is necessary for ER retention of CD4. These complexes have been visualized by immunoelectron microscopy near nuclear pores (Koga *et al.*, 1990a); they may impair transport to the nucleus of a protein that contains a nuclear localization signal (Koga *et al.*, 1991). CD4–gp120 binding can induce conformational changes in gp120 (Sattentau and Moore, 1991) and envelope monomers or misfolded oligomers fail to exit the ER (Hunter and Swanstrom, 1991). Therefore, CD4–gp160 complexes in the ER are likely to cause conformational alterations in gp160 that may prevent proper oligomerization or misfolding of oligomers and impair exit from the ER.

A potential therapeutic strategy is derived from the mutual retention of CD4 and gp160 in the ER (Buonocore and Rose, 1990). Coexpression of gp120 or gp160 with a soluble CD4 molecule, containing the four external Ig domains fused to the sequence Ser-Glu-Lys-Asp-Glu-Leu (i.e., the last four residues comprise an ER retention signal for secreted proteins), inhibited both secretion of gp120 and syncytium formation (Buonocore and Rose, 1990). In addition, HIV infection of a transfected high-level CD4-expressing cell line did not lead to depletion of cell surface CD4 (Marshall *et al.*, 1992). Furthermore, infection of this cell line was abortive, with no syncytium formation taking place. This may be partly explained by the fact that the processing of gp160 in infected T lymphocytes is inefficient (i.e., only 5–15% of total gp160 is cleaved to gp120 and gp41) (Willey *et al.*, 1988a). Thus, overexpression of CD4 may result in more abundant complex formation with gp160, eliminating both processing into mature glycoproteins and virus production.

F. Vpu

The Vpu (virion protein U) gene encodes a small, 81-amino acid protein of 16 kDa that is unique to HIV-1 (Cohen *et al.*, 1988; Strebel *et al.*, 1988). Several laboratory strains of HIV-1 lack functional *vpu* genes due to either mutated initiation codons or premature stop codons (Cohen *et al.*, 1988; Strebel *et al.*, 1988). Thus, the *vpu* product may be dispensable for HIV-1 replication *in vitro*. Vpu is found neither at the cell surface nor in virions (Klimkait *et al.*, 1990; Strebel *et al.*, 1988, 1989); it has a hydrophobic N terminus and appears to be an integral membrane phosphoprotein that localizes to the perinuclear region of

the cytoplasm. Vpu shares certain structural properties, including size, hydropathicity, membrane association, and domain structure, with the influenza virus M2 protein (Klimkait *et al.*, 1990). M2 is a tetrameric transmembrane protein that was reported to be a pH-regulated ion channel that could be blocked by amantadine (Pinto *et al.*, 1992). Infection of T cells by Vpu⁻ mutants of HIV resulted in accumulation of intracellular viral proteins and impaired virion release. However, infectiousness of individual viral particles was not diminished. In addition, infection by Vpu⁻ particles caused more cyto-toxicity and syncytium formation than that associated with Vpu⁺ viruses (Klimkait *et al.*, 1990; Strebel *et al.*, 1988, 1989; Terwilliger *et al.*, 1989; Yao *et al.*, 1992). Transmission electron microscopy revealed both greater numbers of plasma membrane-associated virus and viral budding into cytoplasmic vacuoles in the case of Vpu⁻ viruses (Klimkait *et al.*, 1990), a finding similar to the intracytoplasmic accumulation of viral particles seen in HIV-infected macrophages (Orenstein *et al.*, 1988). This effect of Vpu on virion release is independent of both envelope glycoprotein and CD4 expression (Yao *et al.*, 1992) and suggests that Vpu may facilitate either rates of viral assembly or release.

Finally, Vpu can induce CD4 degradation in the presence of gp160 (Willey *et al.*, 1992a,b). Retention of CD4 in the ER, in the form of gp160 complexes, is essential for this process (Willey *et al.*, 1992b). Vpu-mediated degradation of CD4 was also shown to occur in the absence of gp160 through use of a mutated CD4 with a C-terminal deletion that causes natural ER retention (Shin *et al.*, 1991a) or by treatment of cells with brefeldin A, a compound that blocks protein export from the ER (Willey *et al.*, 1992b). Thus, gp160 functions to retain CD4 in the ER (Willey *et al.*, 1992b); by degrading CD4, Vpu reduces the extent of CD4–gp160 complexes and enhances gp160 processing.

G. Nef

Nef is a 27-kDa, myristylated, cell membrane-associated phospho-protein derived from an open reading frame at the 3′ end of the HIV-1 genome (Fig. 2). The *nef* gene is conserved in both HIV-2 and SIV but is dispensable for HIV-1 replication *in vitro*. Nef is expressed during the early regulatory phase of HIV-1 gene expression, along with Tat and Rev (reviewed in Cullen, 1992b; Greene, 1990, 1991).

Nef was reported to bind GTP and to possess both GTPase and auto-phosphorylation activities (Guy *et al.*, 1987). In addition, HIV-1 Nef⁻ mutants replicated to higher levels than did wild-type viruses. Nef may act as an HIV-1 LTR-specific transcriptional repressor, possibly by inhibiting induction of NF-κB (Niederman *et al.*, 1992). However, these

properties of Nef are controversial and have not been confirmed by others (reviewed in Cullen, 1991b; Greene, 1990, 1991). Allelic variation in the effects of Nef may help to explain these contradictory findings, including ability to retard or accelerate HIV-1 replication (Terwilliger *et al.*, 1991; Zazopoulos and Haseltine, 1993). HIV obtained early in disease was shown to be repressed in cell lines that expressed Nef, whereas viruses from patients with advanced disease were not affected in the same host cells (Cheng-Mayer *et al.*, 1989). The Nef of SIV has also been shown to be dispensable in tissue culture systems, but is essential for viral replication and disease progression in rhesus monkeys (Kestler *et al.*, 1991).

Expression of Nef in several, but not all CD4$^+$ cell lines led to CD4 surface downregulation (Cheng-Mayer *et al.*, 1989; Gama-Sosa *et al.*, 1991; Garcia and Miller, 1991; Garcia *et al.*, 1993; Guy *et al.*, 1987). Nef did not affect either CD4 mRNA or protein levels. Nef also caused cell surface depletion of a cytoplasmic tail triple serine mutant of CD4 (Garcia and Miller, 1991), which cannot be induced to internalize by phorbol esters (see above and Fig. 1). However, the cytoplasmic tail of CD4 was necessary for Nef-mediated CD4 downmodulation, because both a cytoplasmic tail deletion mutant of CD4 and a hybrid CD4/CD8 molecule, containing only the first two N-terminal Ig domains of CD4, were not affected by Nef expression (Garcia *et al.*, 1993).

V. Conclusion

There are, at present, at least three HIV-1 gene products known to be involved in cell surface CD4 downmodulation. These are Nef, Vpu, and gp160. Whereas Nef is expressed during the early phase of HIV-1 gene expression, both Vpu and gp160, which appear to act coordinately, are expressed during the late phase. This functional convergence of HIV-1 proteins on cell surface CD4 downmodulation, whether specific or nonspecific in activity, suggests that this event is of critical importance in the life cycle of HIV-1. Thus, a greater understanding of this phenomenon may provide insights into the interplay between retroviral and host cell proteins involved in immunological responsiveness and signal transduction. Such knowledge could uncover mechanisms that lead to virus-induced cytopathology, that are, in turn, responsible for immunodeficiency.

In addition, further elucidation of the mechanisms that underlie CD4 cell surface downmodulation may lead to the development of novel strategies aimed at preventing such events, and potentially to the development of new therapeutic approaches.

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