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Human immunodeficiency virus infection is efficiently mediated by a glycolipid-anchored form of CD4

(glycosyl-phosphatidylinositol anchor/viral receptor/lipid-anchored protein)

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ABSTRACT **Two broad roles have been revealed for the CD4 molecule. It serves as a receptor for both class II major histocompatibility complex molecules and human immunodeficiency virus (HIV). Upon binding class II major histocompatibility molecules, CD4 functions to enhance T-cell activation. By binding to CD4, HIV gains entry into the cell. We have used a chimeric molecule of CD4 and lymphocyte function-associated antigen 3 (LFA-3), CD4PI, which lacks a membrane-spanning domain and is instead anchored in the membrane by linkage to glycosyl-phosphatidylinositol. To further define the structural attributes of viral receptors, and specifically those of CD4 required for HIV infection, we have expressed CD4PI and CD4 in a human T-cell line, HSB-2. We find that CD4PI is able to mediate infection of these cells by HIV with similar, if not greater efficiency, compared with wild-type CD4. Thus the membrane-spanning region of CD4 is not required for HIV infection, and a lipid-anchored protein can serve as a viral receptor.**

Few viral receptors have been identified and thus information on the general structural features required for their role in viral infection is limited. Similarly, the extent to which their normal function is distinct from or interrelated with their ability to mediate infection is ill-defined. One of the most thoroughly studied viral receptors is CD4, the receptor for human immunodeficiency virus (HIV) (1–3). The natural ligands of CD4 are the class II major histocompatibility complex (MHC) molecules (4). CD4 is most commonly found on T cells restricted by class II MHC molecules and is thought to participate in T-cell antigen recognition, enhancing activation by interacting with a monomorphic domain of class II MHC molecules (for review see ref. 5). Thus, its expression on antigen-responsive T-cell hybridomas has been found to enhance their responsiveness to antigenic stimulation (6, 7).

The binding of gp120, the envelope protein of HIV, to CD4 can disrupt CD4's normal functioning in several ways. gp120 can block CD4 binding to class II MHC molecules (8, 9), inhibit CD4-dependent antigen reactivity (10–12), and perhaps induce inhibitory signals (12). Previous work has shown that alteration or replacement of various cytoplasmic and membrane-spanning regions of CD4, as well as deletions of the cytoplasmic domain, can disrupt modulation by phorbol 12-myristate 13-acetate (PMA) (13–15) but not the inhibitory effects of gp120 (10) or the ability to mediate infection by HIV (13, 14). While these mutants show that the wild-type CD4 sequence of these regions is not required by HIV, they do all have membrane-spanning and, at least, rudimentary cytoplasmic domains, thus leaving open the possibility of a generic requirement for such structures in order to serve as a viral receptor. The retention of membrane-spanning sequences may allow continued interactions with other membrane-spanning regions. Likewise, even the few cytoplasmic amino acids that are retained may allow interactions with cytoskeletal or other proteins at the inner surface of the membrane. Any of these interactions could be important for the uptake of virus, which must follow binding.

Here we demonstrate the ability of CD4PI, a lipid-anchored form of CD4, to mediate productive infection by HIV, thereby proving that neither the membrane-spanning nor the cytoplasmic domain of CD4 is required, specifically or as a structural element, in order for CD4 to function as a viral receptor. These results serve to identify the class of naturally glycosyl-phosphatidylinositol (GPI)-anchored proteins as potential viral receptors.

MATERIALS AND METHODS

Media. All experiments were carried out in RPMI 1640 supplemented with 10% calf serum, 10 mM Hepes, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 units of penicillin per ml, and 100 μg of streptomycin per ml except as noted otherwise. Once established, the HSB2-derived lines were carried in the same medium but with only 5% serum.

Expression of CD4 and CD4PI in HSB-2 Cells. Expression of the CD4 constructs in the human T-cell line HSB-2 was obtained by coculture with the retroviral producer lines MNST4 DAMP (6) and MNCT4PI DAMP as described (6, 15, 16). In brief, the retroviral producer lines were generated by transfection, by calcium phosphate coprecipitation, of the amphotropic helper line DAMP with defective proviral vector DNA carrying neomycin resistance and the cDNA encoding CD4 or CD4PI, followed by selection with the antibiotic G418 (1 mg/ml). Following coculture the HSB-2 infectants were separated from the adherent producer line by repeated plating and subjected to selection with G418 (1–2 mg/ml). CD4- and CD4PI-expressing lines were identified by indirect immunofluorescent staining with the anti-CD4 monoclonal antibody (mAb) OKT4. Some $CD4^+$ lines chosen for initial experiments were subsequently cloned by limiting dilution in 10% HSB-2-conditioned medium.

Phospholipase Treatment. Cells were incubated 1.5 hr at 37°C in RPMI 1640 plus 10 mM Hepes, 50 μM 2-mercaptoethanol, 0.2% bovine serum albumin, and 0.1% sodium azide, with or without phosphatidylinositol-specific phospholipase C (PI-PLC), and subsequently stained. Removal of CD4 was calculated as the percent reduction of mean fluorescence in

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Abbreviations: HIV, human immunodeficiency virus; MHC, major histocompatibility complex; GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; LFA-3, lymphocyte function-associated antigen 3; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate.

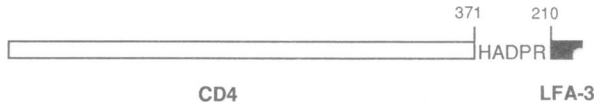

FIG. 1. Structure of the CD4PI construct. The protein encoded by this hybrid gene has authentic CD4 sequence up to amino acid 371 and, following a 5-amino acid insertion (shown in single letter code), the authentic sequence of the GPI-anchored form of LFA-3 from amino acid 210. The hybrid gene consists of CD4 cDNA sequence through the *Fnu*4HI site at base pair 1257 (24), where a *Bam*HI linker was inserted. It then continues, to the end of LFA-3, through a *Bam*HI linker inserted in the *Nsp*BII site at base pair 636 (23).

treated compared with untreated cells after subtraction of background fluorescence in the presence of fluoresceinated antibody alone. PI-PLC from *Bacillus thuringiensis* (17) was the gift of M. G. Low (Columbia University).

Staining and Modulation. Cells were stained in 96-well V-bottom plates, using 50 μl of undiluted OKT4 culture supernatant followed by fluorescein isothiocyanate-conjugated goat $(Fab')_2$ anti-mouse immunoglobulin and were fixed in 1% paraformaldehyde. For modulation experiments the cells were incubated overnight with PMA (30 ng/ml) prior to staining. Fluorescence was assessed by flow cytometry (Epics IV, Coulter Electronics).

Infection. Cells (10^6) of HSB-2, HSBCD4-10, and HSBCD4-PI-7.13 were incubated in 1 ml of HIV-1-infected H9 supernatant ($\approx 10^{11}$ infectious units per ml). H9 is a clone, selected for high and stable production of HIV-1, from the human T-lymphoid leukemia line HT (18). Both the MN and RF strains of HIV-1 have been used. Following incubation with virus for 1 hr at 37°C, the cells were washed twice and put into culture. The cells were washed again 2 days after infection. Supernatants were sampled periodically and assayed for production of the viral p24 core antigen with a commercial ELISA kit (DuPont in Fig. 3*A*; Abbott in Fig. 3*B* and Fig. 4). The maximum capacity of the assay is 500 pg/ml. For antibody blocking experiments cells were first treated with affinity-purified OKT4 or OKT4A (100 μg/ml) and then incubated with virus plus antibody.

RESULTS

Expression and Behavior of CD4 and CD4PI in HSB-2. It has been shown that soluble CD4, which preserves only the extracellular domains of CD4, can bind gp120 (19–22). To determine the role of the membrane-spanning and cytoplasmic domains of CD4 in HIV infection a mutant CD4 molecule, CD4PI, was constructed (B. Seed, unpublished work). CD4PI retains the extracellular domains but lacks the membrane-spanning region as well as the cytoplasmic domain and is instead anchored in the membrane by GPI. The CD4PI gene construct is a hybrid of cDNAs for CD4 (6) and the GPI-anchored form of lymphocyte function-associated antigen 3 (LFA-3) (23) in which the sequences encoding the membrane-spanning and cytoplasmic domains of CD4 have been replaced with those encoding the C-terminal regions of LFA-3 (Fig. 1). The exact position of the posttranslational addition of the GPI anchor and the detailed structure of the GPI anchor itself have yet to be determined for LFA-3. However, the structure of the GPI anchor is similar among those proteins where it is known, although the amino acid linkage and the constituent sugars and lipids do vary (25).

To determine whether a GPI-anchored molecule with the extracellular domains of CD4 was sufficient to serve as a receptor for HIV, we expressed CD4 and CD4PI on the $CD4^-$ human lymphoblastic leukemia line HSB-2 (26). To confirm that this hybrid gene actually confers expression of a GPI-anchored protein, CD4PI was released by treatment with PI-PLC. On the order of 50–70% of CD4PI (expressed on HSBCD4PI-7.13) could be removed by PI-PLC (1:67 dilution) compared with <7% of wild-type CD4 (expressed on HSBCD4-10.28). Incomplete removal of CD4PI by PI-PLC is not surprising. Indeed, incomplete removal of GPI-anchored proteins is the norm. It has been noted that several factors, including state of activation and the particular cell line being used, can affect the susceptibility of GPI-anchored proteins to cleavage (25). In particular, acylation of the inositol ring in some fraction of the protein may prevent release despite cleavage of the phosphate-diacylglycerol bond by PI-PLC, as is the case for acetylcholinesterase (25). Finally, the CD4PI construct was expressed in L cells, which are defective in GPI-anchor biosynthesis. It was previously shown that when Qa2, a protein that is naturally GPI-anchored, was expressed in L cells, none was detected on the cell surface but a soluble molecule was detected in the medium (27). When the CD4PI construct is expressed in L cells, soluble CD4 can be detected in the culture supernatant but no surface expression is observed by flow cytometry (M. Bowman & S.J.B., unpublished observations).

The behavior of CD4 is altered by the substitution of the GPI anchor. In contrast with full-length CD4, which can be modulated from the cell surface by treatment with PMA, the surface expression of CD4PI is increased by PMA treatment (Fig. 2), as is the expression of LFA-3 (data not shown). The increased brightness upon flow cytometric analysis is not due

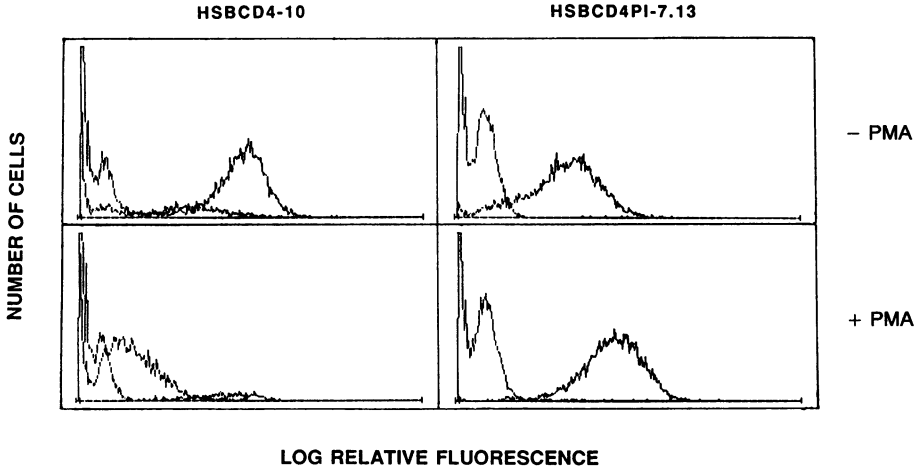

FIG. 2. CD4 and CD4PI expression and effect of PMA. Relative fluorescence by flow cytometry of HSBCD4-10 and HSBCD4PI-7.13 with and without overnight treatment with PMA.

to a PMA-induced increase in cell size as judged by forward-angle light scatter (data not shown). The anti-CD4 mAb Leu-3a, when crosslinked with anti-immunoglobulin, also modulates full-length CD4 but does not modulate CD4PI as detected by immunofluorescent staining (data not shown).

Infection by HIV-1. The ability to mediate HIV infection was tested on CD4PI-expressing HSB-2 (HSBCD4PI-7.13), as well as HSB-2 and wild-type CD4-expressing HSB-2 (HSBCD4-10). Productive HIV infections, detected as the production of p24 core antigen, could be established in both HSBCD4-10 and HSBCD4PI-7.13 but not in the $CD4^-$ parent line HSB-2 (Fig. 3*A*). The earlier detection of p24 core antigen from the HSBCD4PI-7.13 infection was a consistently reproducible observation, suggesting that CD4PI might be a more efficient receptor. Dilution of the virus stock (H9 supernatant) showed that HSBCD4PI-7.13 appeared to be more sensitive to infection than HSBCD4-10 (Fig. 3*B*).

To confirm that infection was indeed proceeding through CD4, infections were carried out in the presence of the anti-CD4 mAbs OKT4A and OKT4, which do and do not block HIV binding, respectively (Fig. 4). OKT4A was able to completely block infection of both cell lines. OKT4 had no effect on HSBCD4-10, though it may have diminished the greater infectibility of HSBCD4PI-7.13.

DISCUSSION

We have expressed a CD4 molecule, CD4PI, that lacks any membrane-spanning or cytoplasmic domain and is instead

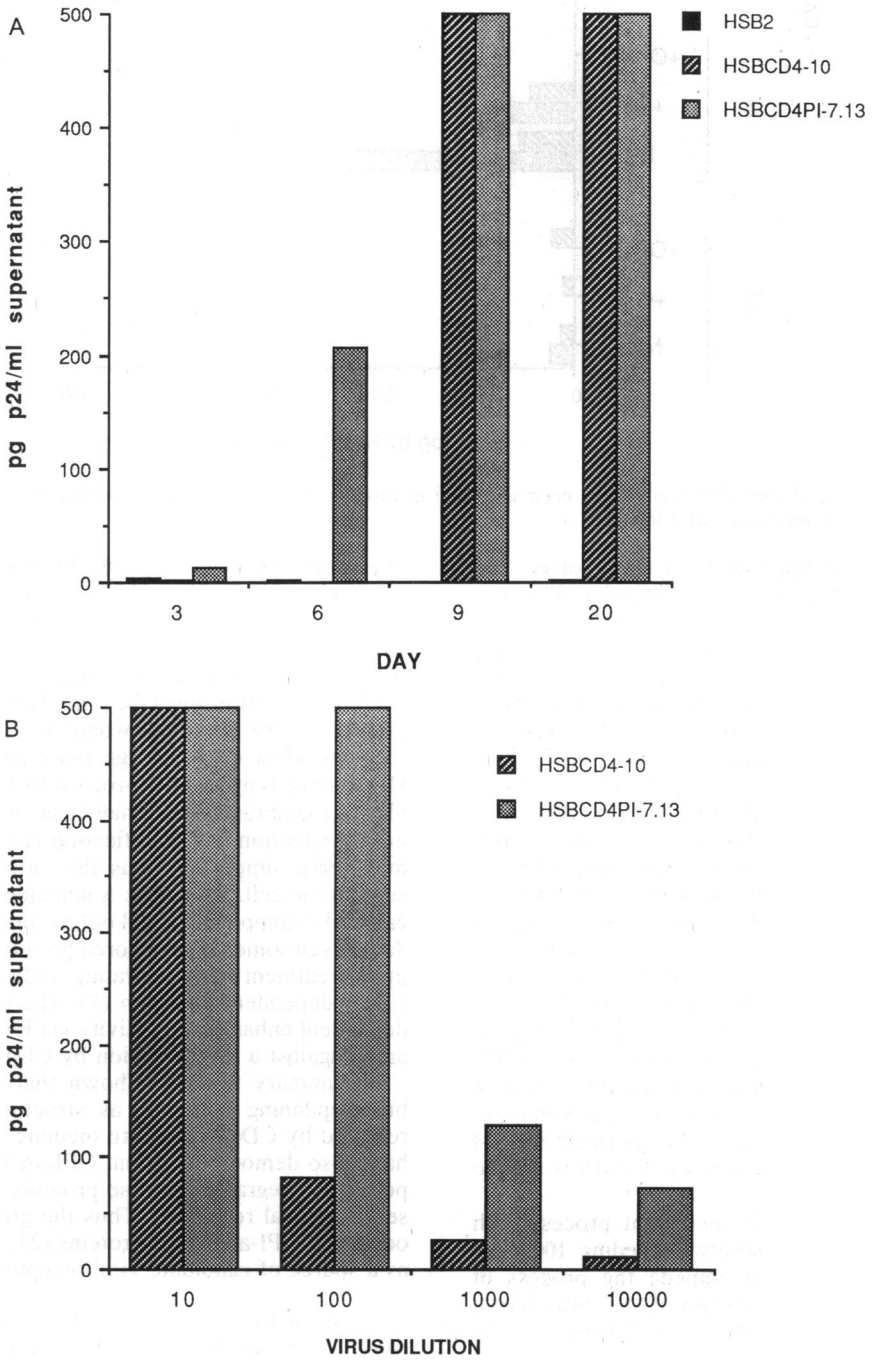

FIG. 3. Infection of HSB lines with HIV. Productive infection was detected as appearance of p24 core antigen. The capacity of the assay is exceeded at 500 pg/ml. (*A*) Time course of core-antigen production at various times after infection. (*B*) Efficiency of infection through CD4 and CD4PI. Infections were carried out using 10-fold serially diluted virus. Results shown are for day 8 after infection.

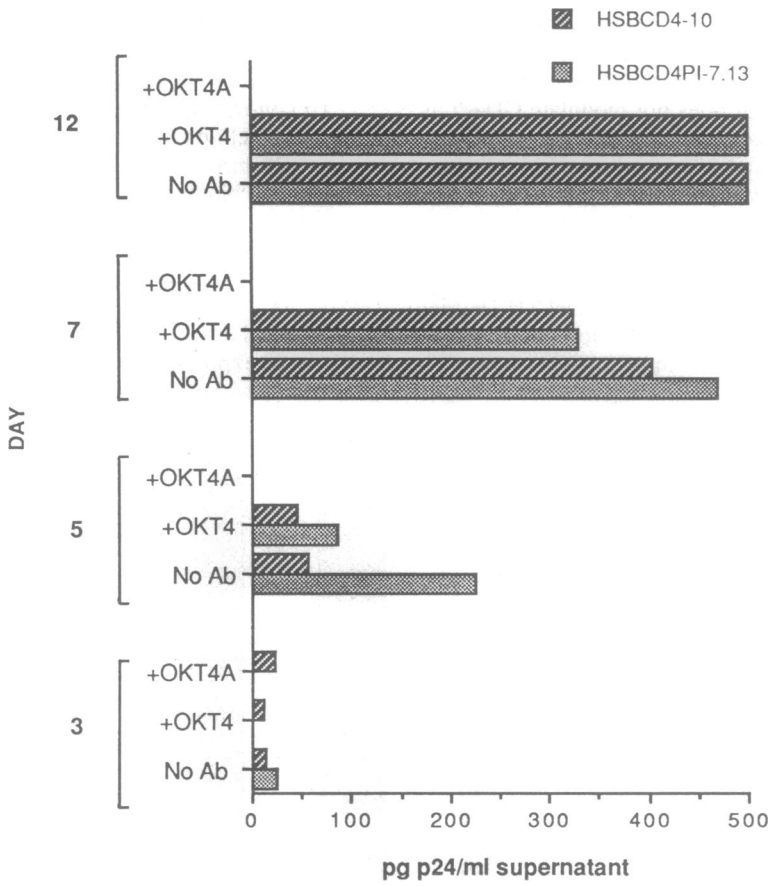

FIG. 4. Antibody blocking of infection. Cells were pretreated and infected in the presence of the indicated antibody and core-antigen production was assayed at various times after infection.

anchored in the plasma membrane by a GPI moiety, and shown that it is still able to mediate HIV infection. Previous work (13, 14) had retained these regions, in whole or in part, respectively, as structural elements. The behavior of a GPI-anchored protein may be expected to differ from integral membrane proteins by having an increased rate of diffusion over the surface (28), though this property is not universal (29). The membrane-spanning region of CD16 (an IgG Fc receptor) interacts with the γ chain of the type I Fc_ε receptor, the presence of which is required for surface expression of this form of CD16 (30, 31), whereas the naturally GPI-anchored form of CD16 is free of this constraint (30–32). While the transmembrane-anchored form of CD16 can transduce biochemical signals, the GPI-anchored form appears to serve only as a binding structure (33, 34). Conversely, when a transmembrane anchor is substituted for the GPI anchor of Qa, it no longer mediates T-cell activation (35). Clearly transmembrane- and GPI-anchored proteins may not be functionally equivalent. Thus it seemed possible that CD4PI might be impaired as a viral receptor as a result of either an increase in mobility, a lost interaction with a possible co-receptor or other protein, or perhaps an impairment in CD4 function. Nonetheless we have found that CD4PI is able to mediate infection by HIV.

Viral infection is a notoriously inefficient process with virion/infectious unit ratios generally exceeding 100. The normal interactions of CD4 may impede the process of infection or direct a significant portion of the virus into a nonproductive pathway. By disrupting these interactions the CD4PI molecule may have released such a constraint or made CD4 more available to whatever co-receptor that might exist. However, as we have not tested an exhaustive number of clones, we cannot rule out some other factor specific to these lines as the explanation of the differing efficiencies of infection. Alternatively, CD4PI may just be more efficient at focusing the virus on the cell. However, this appears unlikely, as CD4 and CD4PI exhibit similar affinities for gp120 (D.C.D., unpublished observations).

Clearly a virion must become bound to the cell surface prior to entry or there would be no requirement for a receptor. How entry is triggered remains unclear. Entry of HIV into cells appears to proceed by fusion directly with the plasma membrane rather than in an endocytic vesicle following invagination and acidification (19, 36, 37). Hence, CD4 may serve simply to focus the virus on the surface of a susceptible cell. However, a dependence on internalization cannot be completely ruled out by any of the experiments to date. Even some GPI-anchored proteins become internalized upon treatment with activating antibodies (38). Reports of CD4-independent infection (39–41), in particular antibody-dependent enhanced infectivity via Fc receptor type III (41), argue against a unique action by CD4.

In summary we have shown that cytoplasmic or membrane-spanning domains, as structural elements, are not required by CD4 in order to mediate infection by HIV. We have also demonstrated that GPI-anchored proteins, as opposed to integral membrane proteins, have the potential to serve as viral receptors. Thus the growing list of naturally occurring GPI-anchored proteins (25, 28) cannot be ignored as a source of candidate viral receptors.

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