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Incorporation of CD4 into Virions by a Recombinant Herpes Simplex Virus

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Two herpes simplex virus type 1 (HSV-1) recombinants were constructed by inserting the human CD4 gene into the HSV-1 genome between the gC promoter and the gC structural gene. These viruses, designated KΔT/CD4 and K082/CD4, synthesized a significant quantity of CD4. CD4 was expressed on the surface of infected cells at levels substantially higher than on the surface of HUT78 cells, a $CD4^+$ cell line. Most significantly, a small but detectable quantity of CD4 was incorporated into virions produced by the recombinant viruses. This was demonstrated both by immunoprecipitation of CD4 from purified virions and by neutralization of the recombinant virions by OKT4 and complement. These results suggest that specific virion incorporation signals are not strictly required for inclusion of glycoproteins into HSV-1 virions. It may be possible to utilize this ability to alter the host range or tissue specificity of HSV-1.

Considerable interest has been expressed in the use of large DNA viruses, including the herpesviruses, as viral vectors (for reviews, see references 19, 24, and 29). Viral vectors are potentially useful for high-level expression of recombinant proteins, as vaccines, and in gene therapy. The use of recombinant viruses for gene therapy will often require the ability to direct or target the virus to specific cells. In effect, this means altering the host range or tissue specificity of a virus.

The herpes simplex virus type 1 (HSV-1) host range is determined, in part, by the ability of the virus to adsorb to and penetrate cells. These functions are carried out by the HSV-1 envelope glycoproteins. Recent results indicate that initial adsorption of HSV-1 to host cells occurs by binding of HSV-1 to cell surface heparan sulfate proteoglycans (32, 39). This interaction appears to be primarily mediated by gC, although gC is not strictly required for virus replication (12, 13, 16). In the absence of gC, adsorption may be mediated by gB (13). Penetration of adsorbed virus requires at least three viral glycoproteins: gB, gD, and gH (1, 2, 7, 23). Other viral proteins may be required as well. How these glycoproteins function to induce membrane fusion is not known. It has been suggested that gD may bind to a second cellular receptor (20), distinct from heparan sulfate, although alternative views of gD function have also been put forth (3).

Conceivably, the host range of HSV-1 could be altered by modification of its array of envelope glycoproteins. The penetration functions of HSV-1 can be abrogated by introduction of lethal mutations into the essential glycoprotein genes (1, 2, 7, 23). However, to redirect the virus to infect a different cell type, the virions of a genetically engineered virus would require one or more foreign glycoproteins able to mediate virion adsorption and penetration of that cell type. Foreign and recombinant genes inserted into herpesviruses can be expressed during the course of viral infection (10, 37, 38). However, the requirements for incorporation of membrane glycoproteins into herpesvirus envelopes are not well understood. The best-studied examples involve the insertion of mutant, foreign, or recombinant glycoprotein genes into pseudorabies virus (PRV) (33, 34, 37, 38). Although some mutant PRV glycoproteins are incorporated into virions, the recombinant and foreign glycoproteins studied were not incorporated. We report here the characterization of recombinant HSV-1 expressing human CD4, a T-lymphocyte surface glycoprotein. CD4 is also the receptor for human immunodeficiency virus type 1, the etiologic agent of AIDS (28). HIV-1 adsorbs to and penetrates $CD4^+$ lymphocytes via the interaction of the HIV-1 *env* (gp120/gp41) glycoprotein with CD4 (25). Virions produced by the recombinant herpesviruses contained CD4, demonstrating that at least certain foreign glycoproteins can be incorporated into HSV-1 virions. We note that CD4 also has been incorporated successfully into both retrovirus and rhabdovirus virions (31, 40). Our findings suggest that the host range or tissue specificity of HSV-1 might be alterable by modification of the virion envelope.

MATERIALS AND METHODS

Cells and viruses. Vero cells were maintained in Eagle minimum essential medium (MEM) supplemented with nonessential amino acids, 50 μg of gentamicin per ml, and 5% fetal bovine serum (FBS). D6 cells and the gB^- mutant viruses KΔT and K082 (1, 2) were obtained from S. Person (University of Pittsburgh). D6 cells were grown in the same medium as Vero cells, with the exception that the antibiotic G418 (Life Technologies, Inc., Gaithersburg, Md.) was added to the medium at a concentration of 500 μg/ml every fourth passage. OKT4 cells (21) were obtained from the American Type Culture Collection (Rockville, Md.). HUT78 cells (9) were obtained from the AIDS Research and Reference Reagent Program (ERC BioSciences Corporation, Rockville, Md.). The wild-type HSV-1 strain KOS321 is a plaque-purified isolate of the KOS strain (15, 16). Stocks of KΔT, K082, KΔT/CD4, and K082/CD4 were produced by infection of D6 cells at a multiplicity of infection (MOI) of 0.1 PFU per cell. Stocks of viruses were concentrated by ultracentrifugation.

Plaque assay. Virus dilutions were made in phosphate-buffered saline (PBS) (5). A total of 0.2 ml of each dilution was applied to a cell monolayer in a 60-mm petri dish and allowed to adsorb for 1 h at 37°C, after which 4 ml of MEM containing 2% FBS and 0.5% methylcellulose was added to

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the dish. The dishes were incubated at 37°C for 3 to 4 days and then stained with crystal violet. The titer of the wild-type strain was determined by plaque assay on Vero cell monolayers. Titers of gB^- mutant virus stocks were determined by plaque assay on both D6 and Vero cell monolayers.

Subcloning. The plasmid pT4.8 was obtained from D. Littman (University of California, San Francisco). This plasmid contains a cDNA clone of the human CD4 gene and is similar to pT4B, which is described by Maddon et al. (26), except that the insert is in the opposite orientation. The plasmid pGC (17) was obtained from F. Homa (The Upjohn Co., Kalamazoo, Mich.). Restriction enzymes and DNA ligase were obtained from Life Technologies, Inc. Enzymes were used according to the directions provided by the manufacturer. A DNA fragment containing the CD4 gene was obtained by digestion of pT4.8 with *Bam*HI and then by preparative electrophoresis in low-melting-temperature agarose (Life Technologies, Inc). The 1.8-kb CD4 fragment was ligated with *Bgl*II-digested pGC, after which the ligation mixture was redigested with *Bgl*II to eliminate recircularized pGC. The DNA was used to transform *Escherichia coli* HB101 as described by Maniatis et al. (27). The plasmids isolated from the resultant colonies were screened for the presence and orientation of CD4 inserts by restriction analysis. A plasmid with the CD4 insert in the correct orientation was designated pCD4/gC.

Marker transfer. Viral DNA was prepared as described by Sandri-Goldin et al. (30). Plasmid DNA was prepared as described by Maniatis et al. (27). D6 cells were transfected by the method of Graham and van der Eb (11) with approximately 30 μg of KΔT or K082 DNA mixed with 20 μg of pCD4/gC DNA. Cultures were harvested when cytopathic effect was generalized. The transfection progeny were titrated on D6 cells and then replated on D6 monolayers in 100-mm petri dishes at approximately 1,000 PFU per dish. Plaque lifts were made from the dishes onto Nytran filter disks (Schleicher & Schuell, Keene, N.H.) (15, 17). A ^{32}P-labeled, CD4-specific probe was produced by using a random oligonucleotide primer labeling kit (Stratagene, San Diego, Calif.). This probe was hybridized to the plaque lifts to identify recombinant plaques. Hybridizing plaques were picked, grown on D6 cells, and subjected to two additional rounds of plaque purification and hybridization screening.

Southern blot analysis. To produce virion DNA, T25 flasks of D6 cells were infected at an MOI of 10. After cytopathic effect became generalized, cells and cell debris were removed from the medium by centrifugation at 2,000 × *g* for 10 min. Virions were pelleted from the supernatant by centrifugation in a SA-600 rotor at 15,000 rpm for 1 h. Virions were resuspended in TE and treated with 20 μg of RNase A per ml at 37°C for 1 h and then digested with proteinase K at 500 μg/ml for 16 h. DNA was extracted from the mixture by phenol-chloroform extraction and ethanol precipitated. Samples (approximately 2 μg) of the extracted DNA were digested with either *Sal*I or *Bam*HI and electrophoresed on a 0.7% agarose gel. After electrophoresis, the DNA was transferred to a Nytran membrane and probed with a ^{32}P-labeled CD4 probe.

Protein labeling and gel analysis. T25 flasks of Vero cells were infected with virus at an MOI of 10 PFU per cell. Four hours after infection, the medium was removed and replaced with MEM containing 1/10 the normal concentration of methionine, 2% FBS, and 30 μCi of [^{35}S]methionine per ml. Cells were labeled until 24 h after infection, at which time the labeling medium was removed and the cells were solubilized with lysis buffer (1% Nonidet P-40, 20 mM Tris [pH 8.0], 50 μg of *Nα-p*-tosyl-L-lysine chloromethyl ketone [TLCK] per ml) containing 5% nonfat dry milk. The lysate was cleared by centrifugation and divided into four parts. To each part was added 10 μl of a pool of monoclonal antibodies specific for gB, gC, or gD (14, 16) or 10 μl of the CD4-specific monoclonal antibody OKT4 (21). The immunoprecipitation reaction mixtures were incubated overnight at 4°C, after which 200 μl of a 10% suspension of protein A beads (Life Technologies, Inc.) was added to the reaction mixtures. The samples were incubated an additional 1 h and then washed six times with lysis buffer. After the final wash, the protein A beads were resuspended in an equal volume of 2× electrophoresis sample buffer and electrophoresed on 10% *N,N'*-diallyltartardiamide-cross-linked gels (22, 35).

Virion purification. Roller bottles of D6 cells were infected with the CD4 recombinant viruses at an MOI of 5, and roller bottles of Vero cells were infected with KOS321 at an MOI of 5. Four hours after infection, the medium was replaced with MEM containing 1/10 the normal concentration of methionine, 2% FBS, and 10 μCi of [^{35}S]methionine per ml. Virions were purified on dextran gradients (36). The purified virions were pelleted in an SA-600 rotor at 15,000 rpm for 1 h and then solubilized with lysis buffer containing 5% nonfat dry milk. Insoluble material was pelleted at 15,000 × *g* for 15 min. The samples were split in two parts, and immunoprecipitations were performed with OKT4 or a pool of gD-specific monoclonal antibodies. The immunoprecipitated proteins were electrophoresed on 10% *N,N'*-diallyltartardiamide-cross-linked gels.

Flow cytometry. Vero cell monolayers were either mock infected or infected at an MOI of 10 with KOS321, KΔT/CD4, or K082/CD4. HUT78 cells were used as a positive control for CD4 expression. Cells were harvested 18 h after infection by using PBS containing 0.5 mM EDTA. After harvesting, subsequent procedures were performed at a temperature of 4°C. The cells were washed and divided into four aliquots, each containing approximately 10^6 cells. The cells were incubated with primary antibody: either OKT4, a gC-specific monoclonal antibody pool, or a gD-specific monoclonal antibody pool, at a concentration of 5 μg/ml in 0.1 ml of antibody incubation buffer (PBS containing 2% FBS and 0.1% sodium azide) for 20 min and then washed with PBS containing 0.1% sodium azide. The cells were incubated with 10 μg of goat anti-mouse immunoglobulin G fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments per ml for 20 min and then washed. The cells were fixed with 1% paraformaldehyde prior to flow cytometry. Cell surface expression of CD4, gC, and gD was measured by flow cytometric analysis with a Coulter EPICS model 753 fluorescence-activated cell sorter.

Virus neutralization. Wild-type virus (produced in Vero cells) and recombinant viruses (produced in D6 cells) were diluted to 5.0 × 10^3 PFU/ml in MEM containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.4) and 20% normal rabbit serum as a source of complement. In a 96-well tray, 100 μl of virus dilution was mixed with an equal volume of monoclonal antibody diluted in PBS and then incubated at 37°C for 1.5 h. After incubation, 150 μl was plated on a D6 cell monolayer in a 24-well tray. After adsorption, medium containing methylcellulose was added to the wells. Plaques were fixed and stained after 2 days. The reciprocal of the dilution of antibody which reduced the plaque count to 50% of the PBS control was considered to be the neutralization titer of the antibody.

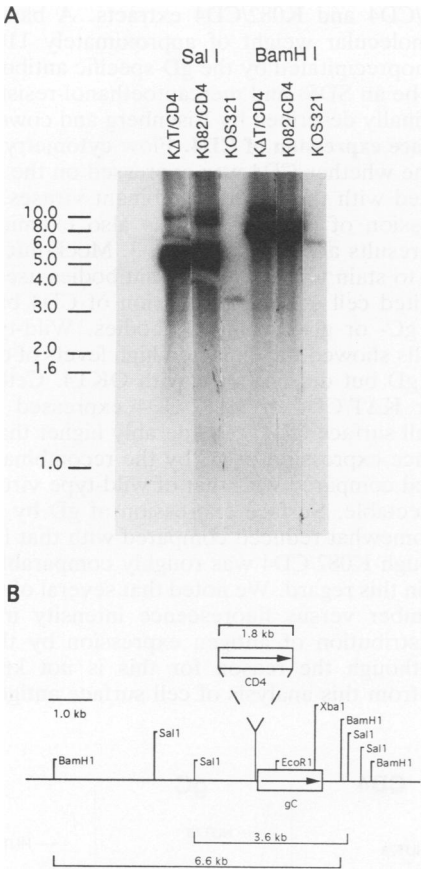

FIG. 1. Recombinant viruses contain the CD4 gene. The CD4 gene was inserted into the gC locus of HSV-1 by marker transfer. Recombinant viruses were identified by plaque hybridization. Virion DNA (approximately 2 μg) from recombinant and wild-type virus was digested with *Sal*I or *Bam*HI, electrophoresed, blotted, and probed with a CD4-specific probe. (A) Autoradiogram of Southern blot. This autoradiogram shows that the digests of recombinant virus DNA contained fragments that hybridized strongly with the probe, while the wild-type digests did not. The sizes of the hybridizing fragments (*Sal*I, approximately 5.4 kb, and *Bam*HI, approximately 8.4 kb) were consistent with insertion of the CD4 gene into the gC locus. (B) Restriction map of the HSV-1 (KOS) genome between coordinates 0.606 and 0.665. The positions of *Sal*I, *Bam*HI, *Eco*RI, and *Xba*I sites are shown. The boxes indicate the positions of the gC and CD4 open reading frames. Arrows indicate the direction of translation. The gC promoter is immediately to the left of the position where the CD4 gene was inserted.

RESULTS

Isolation of recombinant viruses. The plasmid pT4.8 contains a cDNA copy of the human CD4 gene (26). This plasmid was digested with *Bam*HI, and the 1.8-kb DNA fragment containing the CD4 gene was gel purified. The plasmid pGC contains the HSV-1 (KOS) gC transcription unit with an 8-bp *Bgl*II linker inserted at position 128 in the 5′ untranslated region of the gC gene (17, 18). The plasmid pCD4/gC was produced by inserting the 1.8-kb CD4 fragment, oriented in the same direction as the gC gene, into the *Bgl*II site of pGC. This placed the CD4 initiation codon approximately 154 nucleotides downstream of the gC transcription initiation site. The initiation codon of wild-type HSV-1 (KOS) gC is approximately 146 bases downstream of the transcription initiation site (17, 18). Sequences important for gC expression have been reported to be located upstream of the *Bgl*II site insertion (17).

TABLE 1. Plating efficiencies of parental and recombinant viruses

Virus	Titer on following cells:		D6/Vero ratio
	D6	Vero[a]	
KΔT[b]	1.2×10^7	5.0×10^2	2.3×10^4
K082	2.8×10^9	2.3×10^5	1.2×10^4
KΔT/CD4	1.7×10^9	5.0×10^3	3.4×10^5
K082/CD4	2.4×10^9	6.8×10^4	1.5×10^4

[a] Plaques formed on Vero cells are thought to be formed by gB^+ revertants.
[b] The KΔT stock used was an unconcentrated lysate. Other stocks used were clarified lysates concentrated by ultracentrifugation.

The plasmid pCD4/gC was constructed for marker transfer of the CD4 gene into the gC locus of HSV-1 by cotransfection. Two HSV-1 (KOS) mutants, KΔT and K082 (1, 2), were selected as vectors for producing the CD4 recombinant viruses. These two mutants are both unable to express HSV-1 gB and can be grown only in complementing cell lines, such as D6 cells (1, 2). These mutants were chosen as the parental viruses for the CD4 recombinants for two reasons. First, an additional measure of biosafety was provided, since the viruses are unable to replicate in normal cells. Second, since gB is required for HSV-1 membrane fusion, membrane fusion mediated by the interaction of CD4 with HIV-1 *env* might be better observed in the absence of gB (1, 8).

Cotransfection progeny were subjected to three cycles of plaquing on D6 cells and screening by plaque hybridization (15, 17) with a CD4 probe. After isolation and purification, virion DNA was extracted from several prospective recombinant viruses derived from both KΔT and K082. Recombinant and wild-type virus DNAs were digested with *Bam*HI and *Sal*I, electrophoresed on 1% agarose gels, blotted, and probed with a ^{32}P-labeled CD4 probe. The Southern blot showed that the restricted virion DNAs of the prospective recombinants contained intensely hybridizing bands of the expected sizes (Fig. 1). The probe hybridized with an approximately 5.4-kb *Sal*I fragment and an approximately 8.4-kb *Bam*HI fragment from each of the recombinant viruses but not from wild-type virus. The more weakly hybridizing bands above the major bands were the result of partial digestion. DNA from wild-type virus did not contain bands corresponding to the major bands seen in the recombinants. The very weakly hybridizing bands in the wild-type lanes are likely to have been caused by cross-hybridization between viral and probe sequences. These results indicate that recombinant viruses KΔT/CD4 and K082/CD4 contained the CD4 gene inserted into the HSV-1 genome at the gC locus.

To verify that the recombinant viruses retained the gB^- phenotype of the parental viruses, their plating efficiencies were measured on both D6 and Vero cells. The parental viruses are unable to plaque on Vero cells but do plaque on D6 cells (1, 2). The recombinant viruses should behave as do the parental viruses in this respect. As shown in Table 1, the plating efficiencies of the parental viruses and the recombinants were 4 to 5 orders of magnitude lower on Vero cells than on D6 cells, indicating that the recombinants retained the gB mutations of the parental viruses.

Glycoprotein synthesis. To determine whether KΔT/CD4 and K082/CD4 expressed the CD4 glycoprotein after infec-

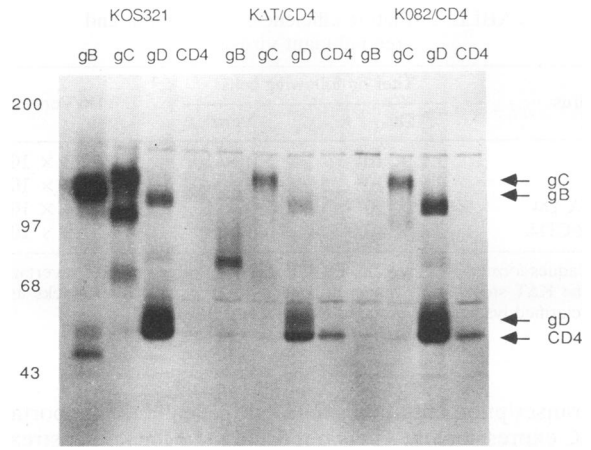

FIG. 2. The recombinant viruses produce CD4. Vero cells were infected with CD4 recombinant (KΔT/CD4 and K082/CD4) or wild-type viruses (KOS321) at an MOI of 10 and labeled with [^{35}S]methionine from 4 to 24 h postinfection. Labeled glycoproteins were immunoprecipitated with monoclonal antibodies specific for CD4 or HSV-1 glycoproteins gB, gC, or gD and then electrophoresed on SDS-polyacrylamide gels. The positions of molecular weight marker proteins are indicated at the side of the gel.

tion, Vero cells were infected with the wild-type strain KOS321, KΔT/CD4, or K082/CD4 at an MOI of 10 and labeled with [^{35}S]methionine. Twenty-four hours after infection, the cells were harvested, and detergent extracts were prepared and clarified by centrifugation. Radiolabeled glycoproteins were immunoprecipitated with monoclonal antibodies specific for gB, gC, gD, and CD4 and then electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels. An autoradiograph of the gel is shown in Fig. 2. The CD4-specific antibody OKT4 immunoprecipitated a band with an approximate molecular weight of 53,000 from extracts of cells infected with KΔT/CD4 and K082/CD4. No such band was immunoprecipitated by OKT4 from cells infected with wild-type HSV-1. The molecular weight of this band is in agreement with the reported molecular weight of human CD4, 55,000 (26). Coincidentally, the apparent molecular weight of CD4 is very similar to the apparent molecular weight of pgD, the precursor form of gD. The OKT4-precipitated band is not pgD, however, since that antibody precipitated nothing from wild-type infected-cell extracts. These results indicate that the recombinant viruses expressed the inserted CD4 gene. Although the inserted gene should be driven by the gC promoter, the quantity of CD4 observed was relatively low, much less than the quantity of gC observed in wild-type HSV-1.

The viral glycoproteins gB, gC, and gD were readily apparent in immunoprecipitates from the wild-type extract. KΔT/CD4, derived from KΔT, synthesized the approximately 78,000 M_r mutant form of gB produced by its parent virus (1, 2). K082/CD4, derived from K082, contains a nonsense mutation near the 5′ end of the gB reading frame and did not produce any detectable form of gB, as has been shown for K082 (1, 2). Interestingly, some gC was immunoprecipitated from both KΔT/CD4 and K082/CD4-infected cell extracts, although the quantity was less than that in KOS321 immunoprecipitates. Thus, insertion of the CD4 gene into the 5′ untranslated region of the gC transcription unit reduced but did not abolish gC expression. Approximately wild-type quantities of gD were immunoprecipitated from KΔT/CD4 and K082/CD4 extracts. A band with an apparent molecular weight of approximately 110,000 was also immunoprecipitated by the gD-specific antibodies. This appears to be an SDS- and mercaptoethanol-resistant dimer of gD, originally described by Eisenberg and coworkers (6).

Cell surface expression of CD4. Flow cytometry was used to determine whether CD4 was expressed on the surface of cells infected with the CD4 recombinant viruses. Cell surface expression of gC and gB was also examined. Flow cytometry results are shown in Fig. 3. Mock-infected Vero cells failed to stain with any of the antibodies used. HUT78 cells exhibited cell surface expression of CD4 but did not stain with gC- or gD-specific antibodies. Wild-type virus-infected cells showed the expected high levels of expression of gC and gD but did not stain with OKT4. Cells infected with either KΔT/CD4 or K082/CD4 expressed very high levels of cell surface CD4, considerably higher than HUT78 cells. Surface expression of gC by the recombinant viruses was reduced compared with that of wild-type virus but was readily detectable. Surface expression of gD by KΔT/CD4 was also somewhat reduced compared with that in the wild type, although K082/CD4 was roughly comparable to wild-type virus in this regard. We noted that several of the curves of cell number versus fluorescence intensity indicated a bimodal distribution of antigen expression by the mutant viruses, although the reason for this is not known. We concluded from this analysis of cell surface antigen expres-

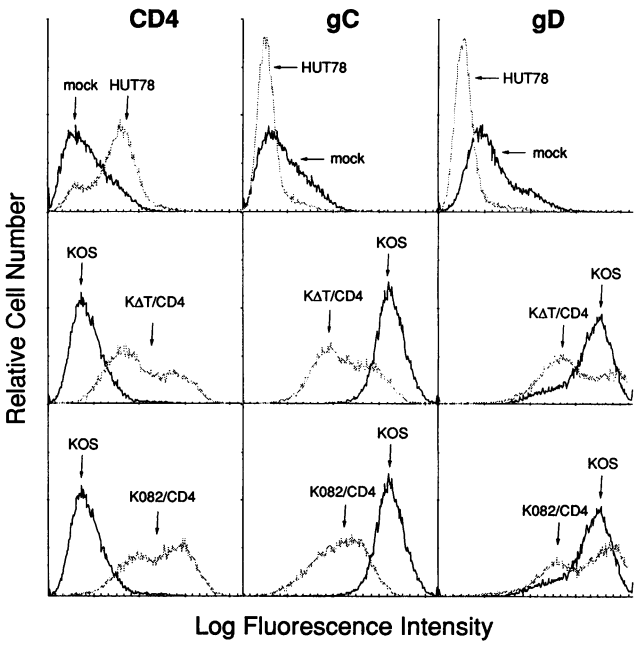

FIG. 3. Cell surface expression of glycoproteins. Vero cells were either mock infected or infected with KOS321, KΔT/CD4, or K082/CD4 at an MOI of 10. The cells were harvested 18 h after infection and immunostained by incubation with monoclonal antibodies specific for CD4, gC, or gD and then by goat anti-mouse immunoglobulin G fluorescein isothiocyanate-conjugated F(ab′)$_2$ fragments. HUT78 cells were used as a CD4$^+$ control. Fluorescently labeled cells were analyzed by using a Coulter EPICS 753 cell sorter. The data are presented as plots of relative cell number versus log fluorescence intensity. Two sets of data are plotted in each graph. Mock-infected Vero cell data are plotted with HUT78 cell data. KΔT/CD4 and K082/CD4 data are plotted with KOS321 (KOS) data.

sion that CD4 expressed by the recombinant viruses was transported to the cell surface, as were gC and gD.

Incorporation of CD4 into virions. Since CD4 is a human protein, it was uncertain whether it would be incorporated into virions during the course of infection with the recombinant viruses. To address this question, dextran gradients were used to purify ^{35}S-labeled extracellular virions from KOS321-infected Vero cells and from KΔT/CD4 and K082/CD4-infected D6 cells. Since only extracellular virions were purified, the possibility of contamination with CD4-containing cellular membranes was minimized. The purified virions were detergent solubilized, and monoclonal antibodies were used to immunoprecipitate gD and CD4 from the extracts. SDS-polyacrylamide gel analysis of the immunoprecipitates (Fig. 4) demonstrated the presence of a small quantity of CD4 in the extracts of KΔT/CD4 and K082/CD4 virions but not in wild-type virions. Approximately equal quantities of gD were immunoprecipitated from each virus extract. Note that no pgD was detected in these virions. Precursor forms of the HSV-1 glycoproteins are found in the nuclear and rough endoplasmic reticulum membranes but are not components of mature virions (4). The absence of pgD from the purified virion preparations suggested that there was little contamination from cellular membranes. The faint band with an apparent molecular weight of 63,000 found in all of the immunoprecipitates is a virion protein that is difficult to remove from the immunosorbent by washing (Fig. 2).

If KΔT/CD4 and K082/CD4 virions contain the CD4 glycoprotein, the virions should be susceptible to inactivation by OKT4 in the presence of complement. Wild-type and recombinant viruses were tested for susceptibility to complement-mediated neutralization by OKT4 and pools of monoclonal antibodies specific for the HSV-1 glycoproteins gC and gD. Significantly, OKT4, in the presence of complement, inactivated the recombinant viruses but not wild-type HSV-1 (Table 2). This confirmed the presence of CD4 in the envelopes of the recombinant viruses. It should be noted that although over 50% of KΔT/CD4 and K082/CD4 virions were inactivated at an OKT4 dilution of 1:2,000, a significant fraction, 15 to 30%, was resistant to neutralization at a dilution of 1:20. HSV-1-specific antibodies inactivated wild-type virus and both recombinant viruses. Neutralization of the recombinants by gC-specific antibodies confirmed the results described above, indicating that the recombinant viruses produced gC.

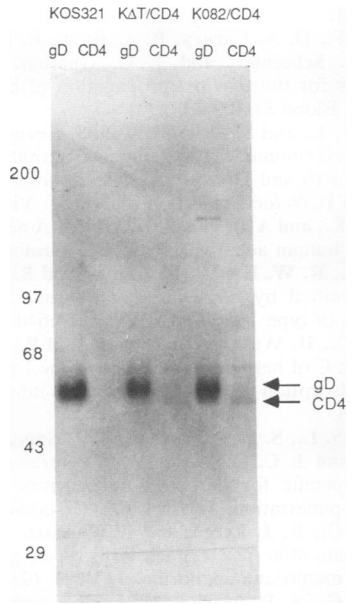

FIG. 4. Recombinant virions contain CD4. Monoclonal antibodies were used to immunoprecipitate gD and CD4 from detergent extracts of dextran gradient-purified, [^{35}S]methionine-labeled KOS321, KΔT/CD4, and K082/CD4 virions. The immunoprecipitated glycoproteins were electrophoresed on SDS-polyacrylamide gels. The positions of molecular weight marker proteins are indicated at the side of the gel.

TABLE 2. Neutralization by glycoprotein-specific antibodies and complement

Virus	50% neutralization titers of antibodies specific for:		
	CD4	gC	gD
KOS321	<20	2,000	200,000
KΔT/CD4	2,000[a]	2,000	200,000
K082/CD4	2,000[a]	2,000	200,000

[a] A 15 to 30% virus survival was noted at lower dilutions.

It was of interest to test whether KΔT/CD4 or K082/CD4 virions could infect cells expressing HIV-1 *env*. We attempted to superinfect several HIV-1-infected lymphocytic cell lines with KΔT/CD4 and K082/CD4. However, we did not detect any cytopathic effect or syncytium formation that might have been due to the recombinant herpesviruses. A number of factors may be responsible for this negative result: the cell lines tested were lymphocytic and may not have supported HSV-1 infection even if penetration had occurred; the recombinant virions may have contained an insufficient amount of CD4 to mediate infection; and finally, factors or conditions in addition to CD4-*env* interaction may be required for membrane fusion to occur. Additional studies on the ability of CD4 expressed by the recombinant viruses to functionally interact with *env* are in progress.

DISCUSSION

We have addressed the question of whether a nonviral glycoprotein can be incorporated into HSV-1 virions by constructing recombinant viruses containing the human CD4 glycoprotein under the control of the HSV-1 gC promoter, a late (γ_2) class promoter (17). CD4 is a typical type I membrane glycoprotein, with an N-terminal signal sequence, a large, glycosylated ectodomain, a hydrophobic transmembrane domain, and a relatively small C-terminal cytoplasmic domain (26). Thus, it is generally similar in structure to several other HSV-1 glycoproteins, such as gC and gD. As a human protein, it presumably lacks any virus-specific targeting signals, if such signals exist. Two recombinant viruses, KΔT/CD4 and K082/CD4, were produced by marker transfer of the CD4 gene into the gC locus of the HSV-1 (KOS) gB$^-$ mutants, KΔT and K082. As with the parental viruses, the recombinant viruses did not plaque on Vero cells but did plaque on the gB-transformed Vero cell line, D6.

Expression of CD4 by the recombinant viruses was readily detected by immunoprecipitation of labeled glycoproteins from infected cells. However, the level of expression was much lower than the expression of the major viral glycoproteins, gB, gC, and gD, by wild-type virus. It is not certain at this time whether the reduction in expression was at the transcriptional or translational level. It had been our expectation that insertion of the CD4 open reading frame between

the transcription and translation initiation sites of gC would reduce gC expression to essentially zero; however, this was not the case. The mechanism by which gC is expressed by the recombinant viruses is unclear; it may involve splicing of a bicistronic CD4/gC transcript or the use of a cryptic promoter within the CD4 gene.

Flow cytometry established that CD4 was expressed on the surface of cells infected with KΔT/CD4 and K082/CD4. In fact, the level of cell surface expression was much higher than that observed on the surface of a $CD4^+$ cell line, HUT78. This may be due to the greater strength of the viral gC promoter compared with that of the cellular CD4 promoter, as well as a greater copy number of the CD4 gene in the infected cells. Thus, although CD4 expression by the recombinant viruses was low compared with gC expression by wild-type virus, they induced levels of CD4 expression much higher than that observed in normal cells. The high level of CD4 cell surface expression and the normal molecular weight of CD4 observed by SDS-polyacrylamide gel electrophoresis suggest that CD4 was posttranslationally processed in the Golgi complex.

Incorporation of CD4 into virions was demonstrated by immunoprecipitation of the glycoprotein from purified virions and by susceptibility of KΔT/CD4 and K082/CD4 virions to inactivation by OKT4 in the presence of complement. To our knowledge, this is the first example of a nonviral glycoprotein becoming incorporated into herpesvirus virions. Since OKT4 did not inactivate 100% of the recombinant virions, some virions may contain little or no CD4. However, the incorporation of CD4 into a majority of KΔT/CD4 and K082/CD4 virions suggests that specific virion incorporation signals are not required for inclusion of glycoproteins synthesized during the infectious cycle into virions. Although it cannot be assumed that the virion formation processes of HSV-1 and PRV are identical, the results presented here are consistent with those of Enquist and coworkers (33, 34, 37, 38). In extensive studies of the PRV gIII glycoprotein, the PRV homolog of gC, they found no evidence for specific virion incorporation signals. By characterizing recombinant PRV expressing mutant PRV glycoproteins, chimeric glycoproteins containing both PRV and foreign glycoprotein sequences, and HSV-1 gC, these workers identified certain factors which correlated with glycoprotein incorporation into virions. These were a relatively high level of expression; Golgi processing; and post-Golgi transport to the cell surface. The characteristics of the HSV-1 CD4 recombinants described here meet these requirements. CD4 expression, although lower than that of the major viral glycoproteins, was nevertheless readily detectable and higher than that observed in HUT78 cells. Synthesis of CD4 of the correct molecular weight suggests that it underwent Golgi processing. A high level of cell surface expression was demonstrated by flow cytometry and is also indicative of Golgi processing. Solomon et al. suggested that glycoprotein incorporation into PRV may be driven by mass action (34). A similar suggestion regarding glycoprotein incorporation into vesicular stomatitis virus was made by Schubert et al. (31). If this is the case, it should be possible to increase the CD4 content of virions by increasing the level of CD4 expression.

As mentioned above, attempts to demonstrate that CD4 in herpesvirus envelopes can interact with HIV-1 *env* on a cell surface to promote membrane fusion have thus far been unsuccessful. This, or a similar demonstration, will be necessary to actually alter the host range of HSV-1 by changing virion glycoprotein composition. Finally, we note that similar studies on the expression, incorporation, and functionality of additional membrane glycoproteins will be required to test the generality of these observations.

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