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Interaction of Glycoprotein H of Human Herpesvirus 6 with the Cellular Receptor CD46*

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Human herpesvirus 6 (HHV-6) employs the complement regulator CD46 (membrane cofactor protein) as a receptor for fusion and entry into target cells. Like other known herpesviruses, HHV-6 encodes multiple glycoproteins, several of which have been implicated in the entry process. In this report, we present evidence that glycoprotein H (gH) is the viral component responsible for binding to CD46. Antibodies to CD46 co-immunoprecipitated an ~110-kDa protein band specifically associated with HHV-6-infected cells. This protein was identified as gH by selective depletion with an anti-gH monoclonal antibody, as well as by immunoblot analysis with a rabbit hyperimmune serum directed against a gH synthetic peptide. In reciprocal experiments, a monoclonal antibody against HHV-6 gH was found to co-immunoprecipitate CD46. Studies using monoclonal antibodies directed against specific CD46 domains, as well as engineered constructs lacking defined CD46 regions, demonstrated a close correspondence between the CD46 domains involved in the interaction with gH and those previously shown to be critical for HHV-6 fusion (*i.e.* short consensus repeats 2 and 3).

Human herpesvirus 6 (HHV-6)[1] is a member of the β-herpesvirus subfamily (reviewed in Ref. 1). Two major HHV-6 variants, A and B, have been defined that form two segregated clusters with unique genetic, biologic, and immunologic characteristics. Primary infection with HHV-6 B occurs almost universally in early childhood and has been etiologically linked to exanthema subitum. In adult life, HHV-6 infection and/or reactivation have been associated with a wide range of diseases, including multiple sclerosis, but most of these associations have yet to be substantiated by rigorous epidemiological studies. In immunocompromised people, including those infected with human immunodeficiency virus, HHV-6 B has been implicated as an opportunistic agent that may cause life-threatening respiratory tract and central nervous system infections, as well as bone marrow or organ graft failure. Moreover, HHV-6 may act as a cofactor to accelerate progression of human immunodeficiency virus disease.

The best studied members of the herpesvirus family enter cells by direct fusion with the plasma membrane in a process involving binding of viral glycoproteins to specific protein receptors on the target cell (reviewed in Ref. 2). Fusion is also facilitated by virus interactions with cell surface glycosaminoglycans. Recently, we identified CD46 (membrane cofactor protein) as a cellular receptor mediating fusion and entry for both HHV-6 variants (3). This glycoprotein also serves as a cellular receptor mediating critical events in the infectious process of other human pathogens, including binding/fusion/entry of measles virus, pilus binding of different pathogenic *Neisseriae*, and adhesion of group A *Streptococci* (reviewed in Ref. 4). CD46 is a member of the regulators of complement activation (RCA) protein family. It is a type I transmembrane glycoprotein of 45–67 kDa, expressed on most or all human nucleated cell types. The N-terminal region consists of four partially homologous short consensus repeats (SCRs) linked in tandem; the SCRs are followed by a serine-threonine-proline (STP)-rich domain, a short region of unknown function, a transmembrane domain, and a cytoplasmic tail. Four distinct CD46 isoforms are variably expressed on different cell types; these contain the same SCRs but differ in the STP and cytoplasmic domains as a result of alternative RNA splicing. Recently the CD46 determinants required for HHV-6 fusion have been analyzed; our results indicated that the critical determinants reside within SCRs 2 and 3, but not SCRs 1 or 4 (5), whereas another study suggested involvement of SCRs 2–4 (6). By contrast, the critical CD46 domains are SCRs 1 and 2 for measles virus, SCR3 and the STP regions for *Neisseria gonorrhoeae*, SCRs 3 and 4 for group A *Streptococcus* (4).

HHV-6 encodes multiple surface glycoproteins, including gB, gH, gL, and gM (1), for which relatively conserved homologs have been identified in all known mammalian herpesviruses. Of these, gH and gL appear to play prominent roles in the membrane fusion events that initiate HHV-6 infectivity, based on inhibitory activities of specific antibodies (7–12). As in other herpesviruses, these glycoproteins form a heterodimeric complex, with gL being required for correct folding, intracellular maturation, and surface expression of gH (9, 12–16). Moreover, gB (the most highly conserved glycoprotein among herpesviruses) and another glycoprotein, gp82-gp105, (thus far found only in HHV-6 and the related β-herpesvirus, HHV-7) appear to be critical for the fusion/entry process, as specific antibodies against these proteins block both virus infectivity and syncytia

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[1] The abbreviations used are: HHV, human herpesvirus; SCR, short consensus repeat; gp, glycoprotein; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; gH, glycoprotein H; DAF, decay accelerating factor; EMEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline.

formation (17–19). Recently the gp82-gp105 glycoprotein has been shown to be associated with gH-gL complexes in infected cells and virions, and the designation gQ was proposed (20). At present, the precise roles of individual HHV-6 glycoproteins and their possible interplay in the infection process remain poorly understood.

In the present study, we sought to identify the HHV-6 glycoprotein(s) that directly interacts with the cellular receptor CD46. Using complementary co-immunoprecipitation approaches, we identified the gH glycoprotein as the CD46-binding component of HHV-6. Mapping studies demonstrated that the CD46 determinants critical for gH binding reside in SCR2 and SCR3, consistent with our previous study (5) indicating the importance of the same CD46 domains for HHV-6 fusion.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—Primary human peripheral blood mononuclear cells (PBMC) were derived from Leukopak preparations obtained from healthy adult donors by gradient centrifugation (BioWhittaker, Walkersville, MD). NIH 3T3 (mouse embryo fibroblasts), RK13 (rabbit kidney cells), and HSB-2 (human immature T-lymphoid cells) were obtained from the American Type Culture Collection. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 10 μg/ml gentamicin). RK13 cells were maintained in EMEM-10 (Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 10 μg/ml gentamicin). HSB-2 cells and PBMC were maintained in suspension in RPMI-10 (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 10 μg/ml gentamicin). Cultures were maintained in a humidified tissue culture incubator at 37 °C in 5% CO_2.

Virus and Infection Procedure—HHV-6 variant A (strain GS) was first propagated in adult human PBMC followed by co-culturing with HSB-2 cells as described (5). Briefly, 20×10^6 PBMC were cultured for 3 days at 2×10^6 cells/ml in RPMI-10 plus 5 μg/ml of phytohemagglutinin (Sigma). Activated cells were centrifuged at $300 \times g$ for 10 min, resuspended in 2 ml of RPMI-10, and infected by incubation for 2 h at 37 °C with cell-free virus stock at an approximate multiplicity of infection of 0.5. The cells were subsequently diluted to 1×10^6 cells/ml and cultured in RPMI-10 for 6–8 days until cytopathic effects were observed. HSB-2 cells were infected with HHV-6 GS by co-cultivation with infected PBMC at a ratio of 5:1.

For vaccinia virus infection, NIH 3T3 or RK13 cells were detached by trypsinization, resuspended at 1×10^7 cells per ml in EMEM with 2.5% fetal bovine serum (EMEM-2.5), and infected with wild-type vaccinia virus Western Reserve (WR) or with recombinant vaccinia virus vCB-48 encoding the BC2 isoform of human CD46 (21) at a multiplicity of infection of 10. After a 2-h incubation at 37 °C, the cells were diluted to 5×10^5 cells/ml in EMEM-2.5 and incubated overnight at 31 °C to allow CD46 expression.

Plasmids and Transfection Procedure—Transfection protocols were performed in EMEM-2.5. Briefly, monolayers of RK13 were singly transfected with plasmids encoding either wild type CD46 (21) or one of the following variants (5): CD46 deletion mutants Δ1-CD46 and Δ2-CD46 lacking SCR1 and SCR2, respectively (22) or CD46-DAF chimeras X3-DAF or X4-DAF (23) (generously provided by Denis Gerlier, CNRS, Lyon, France) in which the SCR3 or SCR4 domains of CD46 were replaced with SCR3 and SCR4 domains of decay accelerating factor (DAF), respectively. All the recombinant CD46 sequences were cloned into pSC59, which contains a strong synthetic vaccinia promoter (24). As a parallel negative control, cells were transfected with the pSC59 empty vector. The transfection procedure was carried out with DOTAP transfection reagent (Roche Applied Science). After 4 h at 37 °C the transfected cells were detached by trypsinization, infected with vaccinia WR, and incubated overnight at 31 °C as described above.

Immunological Reagents—Immunoprecipitation reactions were performed with monoclonal antibodies (mAbs). The following mAbs against human CD46 were used (at 25 μg/ml): J4.48 (25) and E4.3 (26) to SCR1, purchased from Beckman Coulter, Fullerton, CA and Santa Cruz Biotechnology, Santa Cruz, CA, respectively; mAbs M177 to CD46 SCR2 and M160 to CD46 SCR3 (27), kindly provided by Tsukasa Seya, Osaka Medical Center, Osaka, Japan; and mAb GB24, which recognizes SCR4 (Ref. 28 and unpublished data). The following mAbs against HHV-6 glycoproteins were employed: anti-HHV-6 gp116/64/54 against gB (used at 25 μg/ml), purchased from ABI, Columbia, MD, and mAbs 7A2 against gH (ascites fluid, used at 1:2000 dilution) and 2D6 against gp82-gp105 (tissue culture supernatant, used at 1:4 dilution) (29) (kindly donated by Dr. Bala Chandran, University of Kansas). The negative control mAb HA.11 to influenza hemagglutinin (30) (used at 25 μg/ml) was purchased from Covance (Princeton, NJ).

For immunoblot analysis, rabbit polyclonal primary antibodies were employed. For CD46 detection, antibody H-294 was purchased from Santa Cruz Biotechnology. For gH detection, a hyperimmune rabbit antiserum was raised against a short peptide spanning the amino terminus of HHV-6A gH from amino acid 79 to amino acid 93 (sequence: ESLTNYEKRVTRFYE). The N-acetylated synthetic peptide linked to keyhole limpet hemacyanin was used for rabbit immunization (Sigma Genosys, The Woodlands, TX). As the secondary antibody, donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Chemicon, Temecula, CA) was employed.

Co-immunoprecipitation Experiments—Co-immunoprecipitation experiments were performed either with metabolically labeled cells coupled with autoradiographic detection or with unlabeled cells using immunoblot analysis. In the labeling protocol, HHV-6-infected HSB-2 cells at 3 days postinfection were washed twice with methionine-free Dulbecco's modified Eagle's medium and resuspended at 5×10^5 cells/ml with methionine-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum and 40 μCi/ml of [^{35}S]methionine; uninfected HSB-2 cells were labeled in parallel as a negative control. After a 20-h culture at 37 °C, the cells were washed and suspended in methionine-free Dulbecco's modified Eagle's medium. Mixtures were prepared containing 1×10^6-labeled HSB-2 cells and 1×10^6 unlabeled cells (NIH 3T3 or RK13) expressing vaccinia-encoded CD46 (wild type or variants) in a total volume of 200 μl and incubated for 1 h at 37 °C. The cells were washed in cold PBS and solubilized in 200 μl of lysis buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.5% (v/v) Triton X-100. Cell lysates were incubated on ice for 20 min, and cellular debris was removed by centrifugation at $24{,}000 \times g$ for 15 min at 4 °C. The lysates were precleared by adding 50 μl of goat anti-mouse IgG agarose (Sigma) and incubating overnight at 4 °C with rotation. Co-immunoprecipitation from the precleared lysates was performed by adding the indicated anti-CD46 mAb (at 25 μg/ml), incubating for 2 h at 4 °C, adding 50 μl of goat anti-mouse IgG agarose, and incubating for 4 h at 4 °C with rotation. The agarose beads were washed four times with 1 ml of lysis buffer; SDS-PAGE sample buffer containing 5% (v/v) 2-mercaptoethanol was added, and the samples were heated for 5 min at 95 °C. Proteins were separated by SDS-PAGE on 4–20% gradient gels (Invitrogen) and blotted onto nitrocellulose membranes (Protran, Schleicher & Schuell, Keene, NH); immunoprecipitated proteins were detected by autoradiography. Where indicated, direct immunoprecipitation from the metabolically labeled lysate was performed by using a mAb against the designated HHV-6 glycoprotein and processing the samples as described above. For studies involving immunodepletion of specific metabolically labeled HHV-6 glycoproteins, the supernatants recovered from the first immunoprecipitations with the anti-glycoprotein mAbs were subjected to a second identical immunoprecipitation reaction. Only minimal amounts of the glycoproteins were precipitated by the second reaction (data not shown), thus verifying the efficacy of the immunodepletion. The supernatants recovered after immunodepletion were incubated with goat anti-mouse agarose to remove any remaining antibodies and then subjected to co-immunoprecipitation with an anti-CD46 mAb as described above.

For the experiments involving detection of unlabeled proteins by immunoblotting, preparation of cell lysates, co-immunoprecipitation reactions, and SDS-PAGE were performed as described above. The resolved proteins were transferred to nitrocellulose membranes, which were then blocked by a 1-h incubation with PBS/0.1% Tween-20 containing 10% milk. The membranes were then washed and incubated with the designated polyclonal antiserum (see above) in PBS/0.1% Tween-20 containing 5% milk. Rabbit antiserum against the gH peptide was used at a 1:700 dilution; rabbit antiserum to CD46 (H-294) was used at a 1:1000 dilution. After a 1-h incubation, the membranes were washed three times with PBS/0.1% Tween-20 and incubated for 1 h with donkey anti-rabbit antibody conjugated to horseradish peroxidase in PBS/0.1% Tween-20 (dilution 1:5000). After three washes the membranes were subjected to enhanced chemiluminescence detection using the Supersignal West Pico chemiluminescent substrate (Pierce).

RESULTS

Co-immunoprecipitation of Human CD46 with HHV-6-associated Proteins—To identify the HHV-6 protein(s) interacting with CD46, co-immunoprecipitation studies were performed to

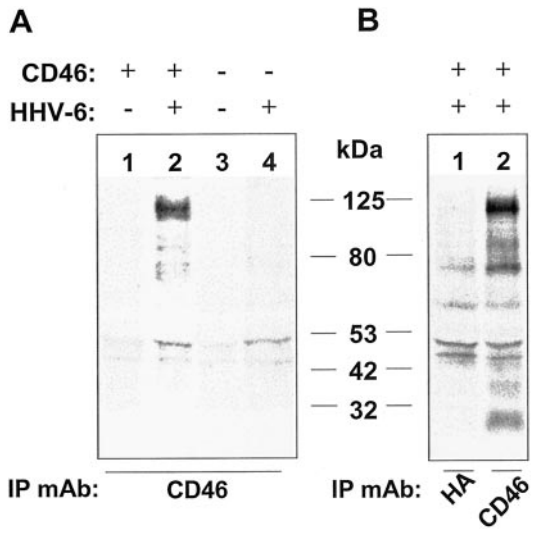

FIG. 1. **A specific HHV-6-associated protein co-immunoprecipitates with CD46.** *A*, immunoprecipitation by an anti-CD46 mAb (J4.48) of proteins from a lysate of [^{35}S]methionine-labeled HHV-6-infected HSB-2 cells and unlabeled NIH 3T3 cells infected with a vaccinia virus expressing human CD46 (vCB48). Uninfected HSB-2 cells and NIH 3T3 cells infected with a control vaccinia virus (WR) served as negative controls. *B*, immunoprecipitation by mAbs against influenza HA (HA.11) or human CD46 (J4.48) of proteins from a lysate of [^{35}S]methionine-labeled HHV-6-infected HSB-2 cells and unlabeled NIH 3T3 cells expressing human CD46. The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography.

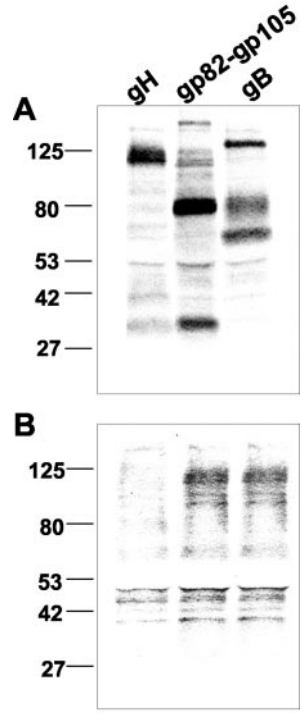

FIG. 2. **Immunodepletion of gH abrogates CD46 co-precipitation of the specific HHV-6-associated protein.** *A*, a lysate from a mixture of [^{35}S]methionine-labeled HHV-6-infected HSB-2 cells and CD46-expressing NIH 3T3 cells was subjected to direct immunoprecipitation by mAbs against the designated HHV-6 glycoproteins. *B*, the supernatant from a lysate of the mixed cells was immunodepleted of the indicated glycoproteins and then subjected to immunoprecipitation with an anti-CD46 mAb (J4.48). The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography.

test the ability of the cellular receptor to form a stable complex with proteins expressed in HHV-6-infected cells. Mouse fibroblasts (NIH 3T3) were infected with a recombinant vaccinia virus encoding full-length human CD46 (BC2 isoform). These cells were mixed with [^{35}S]methionine-labeled human immature T-lymphoid cells (HSB-2) productively infected with HHV-6, strain GS (variant A). Flow cytometry verified surface expression of HHV-6 glycoproteins (gH, gp82–105, and gB). The cell mixture was lysed and subjected to immunoprecipitation with an anti-human CD46 mAb (J4–48); this mAb is specific for the SCR1 domain, which is dispensable for HHV-6 fusion (5, 6). The immunoprecipitates were resolved by reducing SDS-PAGE as described under "Experimental Procedures." As shown in Fig. 1*A*, a major radiolabeled band of ~110 kDa was co-precipitated with anti-CD46 (*lane 2*); this band was absent from a control reaction using uninfected HSB-2 cells (*lane 1*), as well as from another control using CD46-negative NIH 3T3 cells (*lanes 3* and *4*). The latter result demonstrates that it was the recombinant CD46 from the NIH 3T3 cells rather than the endogenous CD46 from the infected HSB-2 cells that mediated the co-immunoprecipitation. Several minor bands were also observed under all conditions and thus were interpreted as background. Further specificity is illustrated in Fig. 1*B*, which demonstrates that the ~110-kDa band was not co-immunoprecipitated by an isotype control mAb HA.11 directed against influenza virus hemagglutinin. Taken together, these results demonstrate that human CD46 interacts with a protein specific for HHV-6-infected cells. The electrophoretic mobility of this protein is consistent with that reported for glycoprotein H (gH) (100–110 kDa, Refs. 9, 10, 14).

Depletion of the CD46-interacting ~110-kDa Protein with an Anti-gH mAb—To assess whether the ~110-kDa band was gH, we tested whether depletion of the supernatant with an anti-gH mAb would result in loss of this band upon subsequent co-immunoprecipitation with anti-CD46. We also examined the effects of depleting two other HHV-6 glycoproteins, gp82-gp105 and gB; all three glycoproteins are known to play critical roles in viral infectivity, as shown by the neutralizing activities of specific antibodies to gH (7–12), gB (17), or gp82-gp105 (19). We first analyzed the products of direct immunoprecipitation from the lysate of a mixture of HHV-6-infected HSB-2 cells and CD46-expressing NIH 3T3 cells (Fig. 2*A*). As expected, the anti-gH mAb (7A2) precipitated a major band of ~110 kDa, which was not observed with the other mAbs. The anti-gp82-gp105 mAb (2D6) precipitated a major protein band of ~80 kDa as well as several minor bands of various sizes, consistent with previous analyses of gp82-gp105 (18, 29). The anti-gB mAb (anti-HHV-6 gp 116/64/54) precipitated three major proteins of ~120, 64, and 54 kDa, consistent with previous analyses of gB (17, 29).

We then analyzed the lysates that had been depleted of specific HHV-6 glycoproteins for co-immunoprecipitation with anti-CD46. With the gH-depleted lysate, the ~110-kDa band was no longer observed, in contrast with the results for the non-depleted supernatants shown above (Fig. 1). However, in both the gp82-gp105-depleted and the gB-depleted lysates, the ~110-kDa band was still observed. These results indicate that all of the detected radiolabeled protein specifically co-immunoprecipitated with anti-CD46 was specifically recognized by the anti-gH mAb.

Confirmation of gH Binding to CD46 by Reciprocal Immunoprecipitation and Immunoblotting—As an additional approach to test for a possible interaction between gH and CD46, we examined the ability of mAbs against one protein to co-immunoprecipitate the other from mixtures of unlabeled HHV-6-infected and CD46-expressing cells; immunoblotting with specific polyclonal antisera was used to detect the co-immunoprecipitated proteins. In Fig. 3*A*, the J4.48 anti-CD46 mAb was used for the initial immunoprecipitation, and a rabbit anti-

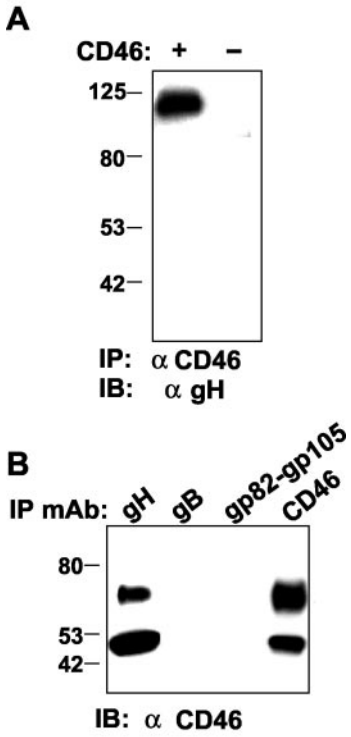

FIG. 3. **Reciprocal co-immunoprecipitation of CD46 and gH, analyzed by immunoblotting.** A lysate from a mixture of unlabeled HHV-6-infected HSB-2 cells and CD46-expressing (or non-expressing) NIH 3T3 cells was subjected to immunoprecipitation with a mAb against the indicated protein. The immunoprecipitates were analyzed by immunoblotting using rabbit antiserum against the designated protein and donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase. *A*, immunoprecipitation was performed with the anti-CD46 mAb J4.48, and immunoblotting was performed with rabbit anti-gH antiserum. The NIH 3T3 cells either expressed (+) or did not express (−) CD46. *B*, immunoprecipitation was performed with a mAb against the indicated HHV-6 glycoprotein (or against CD46), and immunoblotting was performed with anti-CD46 antiserum. *IP*, immunoprecipitation; *IB*, immunoblot.

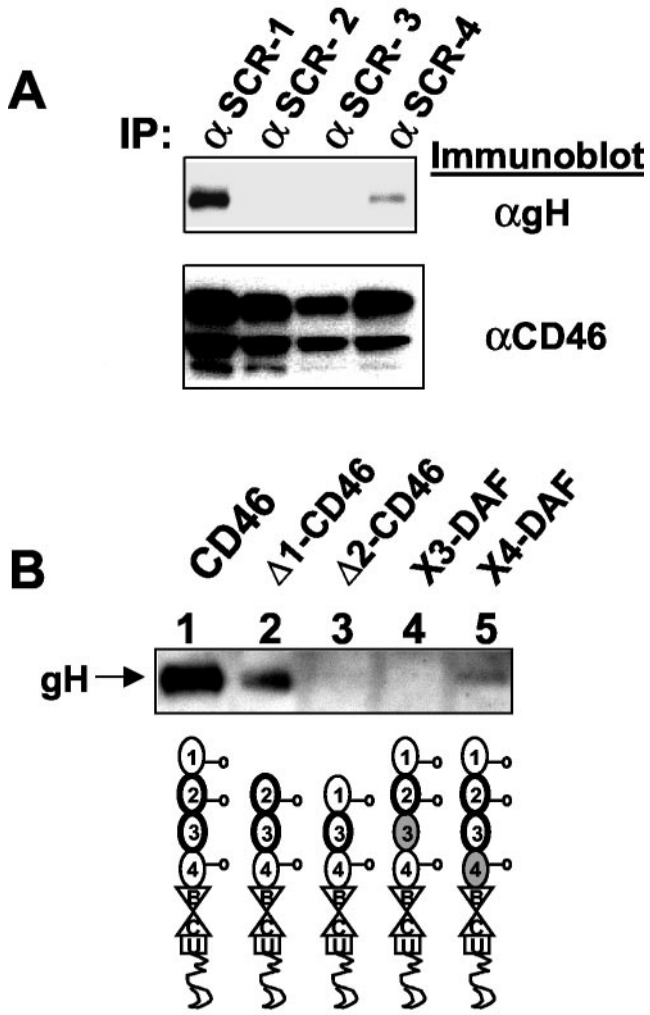

FIG. 4. **Importance of CD46 SCR2 and SCR3 for gH binding.** *A*, immunochemical mapping. A lysate from a mixture of HHV-6-infected HSB-2 cells and CD46-expressing 3T3 cells was subjected to immunoprecipitation (*IP*) by mAbs against the indicated regions of CD46. The immunoprecipitates were analyzed by immunoblotting with antiserum against gH (*upper panel*). The blots were then stripped and analyzed with antiserum against CD46 (*lower panel*). *B*, molecular genetic mapping. RK13 cells expressing CD46 or the indicated molecular variant were mixed with HSB-2 cells infected with HHV-6. The cell lysate was subjected to immunoprecipitation by an anti-CD46 mAb, and immunoblotting was performed with antiserum against gH. Because of the varying presence of different domains in the CD46 constructs, the appropriate anti-CD46 mAb was used for immunoprecipitation in each case; GB24 that recognizes SCR4 was used for Δ1-CD46 and E4.3 against SCR1 was used for the other constructs. The immunoprecipitates were analyzed by immunoblotting with antiserum against gH. *Bolded* SCRs indicate domains previously shown to be required for HHV-6 fusion to target cells (5). *Shading* denotes domains derived from DAF.

serum prepared against a synthetic peptide corresponding to residues 79–93 of gH from HHV-6 strain U1102 (variant A) was used for detection of gH (the specific reactivity of this antiserum for gH was verified by immunoblotting; data not shown). A single prominent ~110-kDa band was observed. This band was absent from a negative control immunoprecipitate from a mixture lacking CD46-expressing cells, thereby verifying that the gH co-immunoprecipitation was mediated by the recombinant CD46 from the 3T3 cells rather than the endogenous CD46 from the HSB-2 cells. This experiment verifies that gH is co-immunoprecipitated with anti-CD46.

A reciprocal experiment was performed to test whether mAbs against different HHV-6 glycoproteins could co-immunoprecipitate CD46 from a lysate of HHV-6-infected HSB-2 cells mixed with recombinant CD46-expressing NIH 3T3 cells. Fig. 3*B* shows that two broad CD46 bands of ~67 and 50 kDa were co-immunoprecipitated by the anti-gH mAb, similar to the profile of bands directly immunoprecipitated by an anti-CD46 mAb. These bands were absent in a control experiment with NIH 3T3 cells lacking recombinant CD46 (data not shown), again demonstrating that they represented the recombinant CD46 rather than endogenous CD46 on the HSB-2 cells. The sizes of the two CD46 bands are consistent with the previously described processing of the high mannose-containing precursor (lower band) to the mature product (upper band) (31). These two bands were previously observed for vaccinia-encoded CD46 (21) and presumably reflect incomplete processing under conditions of vaccinia-mediated overexpression.

Importantly, Fig. 3*B* also shows that the co-immunoprecipitation of CD46 was specific for gH, because it was not observed with the anti-gB mAb or the anti-gp82-gp105 mAb.

Correlation between the CD46 Domains Involved in gH Binding and HHV-6 Fusion—In our previous report (5) on the CD46 regions required for HHV-6-mediated cell fusion, a critical role was observed for the two central SCR domains, SCR2 and SCR3. To test for a possible relationship between these two regions of CD46 and the co-immunoprecipitation of gH, two types of experiments were performed. Fig. 4*A* shows gH co-immunoprecipitation studies performed using mAbs directed against each of the four SCR domains of CD46. Marked differences were observed (Fig. 4*A*, *upper panel*): co-immunoprecipi-

tation occurred with the mAbs directed against SCR1 or SCR4 but not with those directed against SCR2 or SCR3. This distinction was noted despite the ability of all the mAbs to directly immunoprecipitate CD46 (Fig. 4*A*, *lower panel*).

As a complementary approach, we tested gH co-immunoprecipitation by variant CD46 constructs differing in their SCR domain compositions. RK13 cells were transfected with plasmids encoding either wild-type CD46, CD46 deletion mutants lacking SCR1 or SCR2, or CD46-DAF chimeras in which SCR3 or SCR4 were replaced with the corresponding domains of DAF. Cells transfected with the empty plasmid vector served as a negative control. After transfection, the cells were infected with a wild-type vaccinia virus to drive expression of the recombinant CD46 molecules. Flow cytometry analysis confirmed equivalent expression of all constructs (Ref. 5 and data not shown). As shown in Fig. 4*B*, gH was co-immunoprecipitated by wild-type CD46 or by CD46 constructs containing both the SCR2 and the SCR3 domains. By contrast, no gH co-immunoprecipitation was seen with CD46 constructs lacking either SCR2 or SCR3 (or in the negative control without CD46, data not shown). Taken together, the results in this section demonstrate a strong correlation between the CD46 domains involved in co-immunoprecipitation of gH and those previously shown to be critical for HHV-6 cell fusion (5).

DISCUSSION

With the rapidly advancing knowledge on herpesvirus entry, the viral glycoproteins responsible for binding to specific protein receptors have been identified in several cases. The best characterized examples are certain members of the α-herpesvirus subfamily, including human herpes simplex viruses 1 and 2 (HHV-1 and HHV-2, respectively), porcine pseudorabies virus, and bovine herpesvirus. These viruses use several alternate receptor proteins, designated HveA, HveB, HveC, and related proteins (2, 32); viral glycoprotein D (gD) has been directly implicated in receptor binding (33–36). However, a gD homolog is not present in some human α-herpesviruses (Varicella Zoster virus, HHV-3) nor is it present in the human γ-herpesviruses (Epstein-Barr virus, HHV-4; Kaposi's sarcoma-associated herpesvirus, HHV-8) or the human β-herpesviruses (human cytomegalovirus, HHV-5, HHV-6, HHV-7). In these cases, a different viral glycoprotein presumably fulfills the role of binding to the primary cellular receptor. For Epstein-Barr virus, entry requires the interaction of the viral gp42 with HLA class II molecules on the target cell surface (37, 38). The viral gp350/220 glycoprotein mediates attachment to cellular CD21 (39, 40), but this activity is not absolutely required for fusion mediated by Epstein-Barr glycoproteins (41).

In this report, we use reciprocal approaches to demonstrate co-immunoprecipitation of the HHV-6 gH glycoprotein with CD46. Based on experiments using mAbs directed against specific CD46 domains as well as CD46 constructs lacking specific domains, we conclude that the critical CD46 determinants for co-immunoprecipitation lie within the SCR2 and SCR3 regions. These results parallel our previous report showing that the determinants essential for HHV-6-mediated cell fusion are contained within the same domains (5). Although our experiments were performed only with HHV-6A, the close homology (~95%, Ref. 42) and immunological cross-reactivity (10) between gH of HHV-6 A and B as well as the shared use of CD46 as a receptor by both variants (3) suggest identical involvement of this glycoprotein in the CD46 interaction for both HHV-6 A and B.

The simplest interpretation of our results is that HHV-6 fusion/entry involves direct binding of the viral gH glycoprotein to CD46, specifically with the SCR 2 and 3 domains. However, our findings must be considered in terms of the well known association of HHV-6 gH with gL (9, 12–16) and the recent demonstration that these glycoproteins form a trimolecular complex with gp82-gp105, in particular with a minor 80-kDa species representing the mature form with complex *N*-linked oligosaccharides (20). Moreover, subsequent binding studies with a soluble CD46 construct suggested that the receptor associates with the gH/gL/gp82-gp105 trimolecular complex (43). These reports raise the question whether our results reflect direct binding of gH to CD46 or indirect co-immunoprecipitation of gH because of its association with gL and gp82-gp105. In the latter case, the CD46 binding site could reside in one of these other glycoproteins or in a site created by the trimolecular complex.

Despite these ambiguities, we believe our data favor a model of direct gH binding to CD46. In the [^{35}S]methionine metabolic labeling experiment shown in Fig. 1, gH was the only labeled band detected upon CD46 immunoprecipitation from infected cells; no band corresponding to gp82-gp105 was observed. This result cannot be explained by inefficient labeling of gp82-gp105 relative to gH, because the two proteins have the identical number of methionine residues (22) and very similar numbers of total residues (706 and 696, respectively). Therefore, a large molar excess of gH relative to gp82-gp105 would have to be invoked. Moreover, immunodepletion of gp82-gp105 did not prevent subsequent co-immunoprecipitation of gH with CD46 (Fig. 2*B*). These results argue strongly against the involvement of gp82-gp105 in CD46 binding. The case against gL is not quite as compelling, also considering the lack of specific immunologic reagents directed against this glycoprotein. The co-immunoprecipitation of a band of the expected mobility of this glycoprotein (~30 kDa) was observed only inconsistently (*e.g.* absent in Fig. 1*A*, present in Fig. 1*B*); based on the presence of four methionine residues in gL, its detection relative to gH would have been expected in both experiments if it were involved in CD46 binding. The issue could be most definitively resolved by binding experiments with individual recombinant HHV-6 glycoproteins. However, because of negative results, such efforts have thus far been inconclusive. Thus, a soluble CD46 construct was reported not to bind to a soluble form of gp82-gp105 or to cells co-expressing recombinant gH plus gL (43). In preliminary experiments,[2] CD46 could be co-immunoprecipitated by recombinant epitope-tagged gH but only when it was expressed in HHV-6-infected cells; the requirement of gL for proper folding and surface expression of gH is a likely contributor to this result.

For those α-herpesviruses in which gD performs the receptor-binding function, gH homologues are still required for fusion/entry, suggesting that gH plays an additional role(s) yet to be determined (2, 32). For HHV-6, which lacks gD, it is reasonable to speculate that gH performs a dual function, *i.e.* this still undefined role(s) plus the receptor binding function. This notion is consistent with the fact that an anti-gH mAb that co-precipitates CD46 (Fig. 3) also blocks HHV-6 fusion/entry (3), suggesting that it interferes with another gH function besides receptor binding. Indeed, there is ample precedent for viral glycoproteins that perform multiple functions, including receptor binding, and for which mAbs exist that neutralize entry/fusion without blocking binding to the receptor protein, *e.g.* the gp120 subunit of human immunodeficiency virus envelope glycoprotein (44). Our results raise the possibility that for other herpesviruses lacking gD, gH might have dual function in entry, including receptor binding. Also of interest are the findings that for some gD-containing α-herpesviruses, gD-deleted variants have been selected that have a restored ability to

[2] F. Santoro, H. L. Greenstone, P. Lusso, and E. A. Berger, unpublished information.

spread by cell-cell fusion; mutations in gH are associated with this (45, 46). Perhaps the modified gH has acquired receptor binding activity, although this has not yet been demonstrated.

The identification of a role for gH in CD46 binding may have implications for specific antiviral strategies as well as for possible clinical exploitation of viral immunomodulatory mechanisms. Because gH plays a central role in the HHV-6 entry pathway and is highly conserved between the two HHV-6 variants, vaccination against this glycoprotein is likely to elicit neutralizing antibodies that inhibit both virus-cell and cell-cell fusion mediated by the viral envelope, as can be inferred from the neutralizing activity of anti-gH antibodies against both processes (3, 7, 10) Moreover, solving the structure of a gH-CD46 soluble complex could provide valuable insight for the rational design of specific inhibitors of HHV-6 entry. Finally, it was recently observed that HHV-6 abrogates the production of Interleukin-12,[3] an effect most likely mediated by engagement of and signaling through CD46, as was previously demonstrated for measles virus (47). Because Interleukin-12 is a critical cytokine in the generation of Th1-polarized immune responses, HHV-6 gH or derivative molecules with reduced immunogenicity might function as specific immunomodulatory agents for suppressing Th1 responses in clinical conditions associated with aberrant T-cell activation, such as connective tissue disorders, type I diabetes, etc., or in the prophylaxis of transplant rejection. Also of interest in this regard is the recent report (48) demonstrating that cross-linking of CD46 on peripheral blood T cells induces a T-cell regulatory pathway featuring secretion of high amounts of Interleukin-10.

The specific functions of individual HHV-6 glycoproteins and the mechanisms by which their interplay promotes membrane fusion represent major areas for future investigation. Undoubtedly, progress would be greatly facilitated by the development of a system in which HHV-6 fusion activity is reconstituted by co-expression of the required combination of glycoproteins, as has been achieved for herpes simplex viruses 1 (49, 50) and 2 (51), Varicella Zoster virus (52), Epstein-Barr virus (41), and HHV-8 (53). Such studies will likely provide detailed mechanistic insights into the HHV-6 fusion/entry process and guide the development of antiviral strategies.

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Interaction of Glycoprotein H of Human Herpesvirus 6 with the Cellular Receptor CD46

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