We employed a quantitative cell fusion assay to identify structural domains of CD46 required for its function as a receptor for human herpesvirus 6 (HHV-6). We examined the activities of recombinant variants of CD46, including different isoforms as well as engineered truncations and molecular chimeras with decay-accelerating factor, a related protein in the family of regulators of complement activation (RCA). We observed strong receptor activity for all four CD46 isoforms, which differ in the membrane-proximal extracellular and cytoplasmic domains, indicating that the critical determinants for HHV-6 receptor activity reside outside the C-terminal portion of CD46. Analysis of the short consensus repeat (SCR) regions that comprise most of the extracellular portion of CD46 indicated a strong dependence on SCRs 2 and 3 and no requirement for SCRs 1 or 4. Fusion-inhibition studies with SCR-specific monoclonal antibodies supported the essential role of SCRs 2 and 3 in HHV-6 receptor activity. These findings contrast markedly with fusion mediated by measles virus glycoproteins for which we observed a strict dependence on SCRs 1 and 2, consistent with previous reports. These results expand the emerging notion that CD46 and other members of the RCA family are co-opted in distinct manners by different infectious pathogens.

Human herpesvirus 6 (HHV-6) is a recently discovered member of the β-herpesvirus subfamily, for which two major subgroups (A and B) have been identified (reviewed in 1–3). Both subgroups preferentially replicate in CD4 T lymphocytes, although they can infect, productively or nonproductively, a broader range of human cell types. HHV-6 B is virtually ubiquitous in the adult human population worldwide and is the cause of exanthema subitum, a febrile disease of infancy which is usually benign but may be accompanied by neurologic complications. Under conditions of immunosuppression, HHV-6 can cause serious opportunistic infections, and may have direct immunosuppressive effects. Moreover, HHV-6 infection may act as a cofactor to accelerate human immunodeficiency virus (HIV) disease progression. Consistent with the ability of HHV-6 to infect the central nervous system, the virus has been suggested to play a role in multiple sclerosis, although this notion remains controversial (4).

Like other well studied herpesviruses (5), HHV-6 appears to enter target cells by a process involving direct pH-independent membrane fusion between the virion envelope and the plasma membrane. Infection can be mediated by both virion/cell and cell/cell fusion. We recently demonstrated that human CD46 (also known as membrane cofactor protein) is a major cellular receptor for HHV-6 (both subgroups A and B) (6). Our conclusions were based on analyses of virus entry/inf ectivity as well as the related process of HHV-6 glycoprotein-mediated cell fusion; in both types of assays, expression of recombinant CD46 rendered nonpermissive cells susceptible to HHV-6 entry and cell fusion (gain of function) and specific CD46 blocking agents inhibited both processes in target cells endogenously expressing CD46 (loss of function).

CD46 is a member of a family of glycoproteins called regulators of complement activation (RCA); these proteins serve to protect autologous host cells from complement-mediated lysis (reviewed in 7, 8). The human genome encodes at least 6 RCA glycoproteins, some membrane-associated and others fluid-phase. Most are characterized by the presence of partially homologous short consensus repeats (SCRs). Each SCR motif contains ~60 amino acids including 4 cysteines that form two conserved disulfide bonds, as well as a few additional conserved residues. The SCRs are tandemly linked beginning at the N terminus. CD46 is a 57- to 67-kDa type I transmembrane glycoprotein expressed on most or all human nucleated cell types; it regulates complement activity by binding C3b or C4b and promoting their proteolytic cleavage by plasma serine protease factor I. Most of the extracellular portion of CD46 is composed of 4 SCRs (1–4; SCRs 1, 2, and 4 contain sites for N-linked glycosylation). The SCRs are followed by a region rich in serine, threonine, and proline (STP region, containing sites for O-linked glycosylation), a small region of unknown significance, a transmembrane domain, and a cytoplasmic tail. Four distinct CD46 isoforms generated by alternative RNA splicing are expressed differentially in various cell types; all contain the same SCRs but differ in the STP and cytoplasmic regions. Decay accelerating factor (DAF, also designated CD55) is another membrane-associated RCA protein widely distributed on different cell and tissue types; it regulates complement activity by promoting the irreversible dissociation of complement convertases. Like CD46, DAF contains four SCRs followed by an STP region, and is attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor.

CD46, DAF, and some other RCA proteins have been exploited by diverse viral and bacterial pathogens (recently reviewed in 7–10). The most extensively studied interaction is the

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‡ The abbreviations used are: HHV-6, human herpesvirus 6; RCA, regulators of complement activation; SCR, short consensus repeat; STP, serine-threonine-proline-rich; MV, measles virus; HA, hemagglutinin; F, fusion glycoprotein; DAF, decay accelerating factor; GPI, glycosylphosphatidylinositol, mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell.
CD46 Domains Required for HHV-6 Receptor Function

use of CD46 as a binding and entry receptor for certain strains of measles virus (MV), first reported in 1993 (11, 12). Subsequent studies focused on identifying critical domains of CD46 required for MV receptor function, using functional assays of viral infectivity and/or cell fusion as well as binding assays of MV virions or hemagglutinin (HA) (13–20). The results point to a critical role of CD46 SCR1 and 2 for MV receptor activity.

In the present report, we examined the CD46 determinants required for HHV-6 receptor function. We used a quantitative cell fusion assay to test the functionality of the different CD46 variants (isoforms or recombinants), as well as the blocking effects of mAbs directed against defined CD46 domains. Our results highlight major differences in the CD46 determinants necessary for HHV-6 versus MV receptor activity.

EXPERIMENTAL PROCEDURES

Cells—Primary human peripheral blood mononuclear cells (PBMCs) were derived from leukopak preparations obtained from healthy adult blood donors by gradient (BioWhittaker, Walkersville, MD) centrifugation. HSB-2, RK13, HeLa, and NIH 3T3 cells were obtained from the American Type Culture Collection. PBMCs and HSB-2-T–lymphocytes were maintained in suspension in RPMI 1640 medium containing 10% fetal bovine serum (inactivated for 45 min at 56 °C), 1% L-glutamine, and gentamicin (10 μg/ml). RK13 cells were maintained in Eagle’s medium containing 10% fetal bovine serum, 1% L-glutamine, and gentamicin (10 μg/ml). HeLa and NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1% L-glutamine, and gentamicin (10 μg/ml).

Generation of Effector Cells Expressing Surface Viral Glycoproteins—Effector cells expressing the desired viral surface glycoproteins were generated either by using infected cells (HHV-6) or by using vaccinia recombinants encoding the desired glycoproteins. In all cases, the effectors also contained bacteriophage T7 RNA polymerase encoded by vaccinia recombinants encoding the desired glycoproteins. In all cases, the effector cells expressing the designated glycoproteins, and the fusion assay was performed as described above.

Flow Cytometry Analysis—The relative cell-surface expression levels of CD46 and related constructs were determined by flow cytometry. 0.5 × 10⁶ cells were suspended in 100 μl of medium and incubated 10 min at room temperature with the CD46 mAb J4.48 at 10 μg/ml; in experiments examining expression of constructs lacking CD48 SCR 1, a suitable mAb against a common SCR was employed. Cells were washed with 3 ml of phosphate-buffered saline containing 1% fetal bovine serum and suspended in 100 μl of this buffer. The cells were treated with 1 μl of anti-mouse phycoerythrin (Beckman Coulter, Fullerton, CA) and incubated for 10 min at room temperature. Cells were washed and resuspended in 0.5 ml of 2% formaldehyde. Fluorocytometric analysis was performed using a FacScan analyzer (BD Biosciences). 5,000 events were accumulated for each sample.

RESULTS

HHV-6 Receptor Activity of Different CD46 Isoforms—We compared the ability of each of the four CD46 isoforms (BC1, BC2, C1, and C2) to function as a receptor for HHV-6. These differ in both their STP regions (B and C, or just C) and cytoplasmic tails (1 or 2). Each isoform was transiently expressed in RK13 cells using vaccinia-based transfection/infecion technology. Flow cytometry was used to measure cell-surface expression on RK13 cells. As shown in Fig. 1, similar expression levels were observed for all four isoforms. Immuno-blot analysis also confirmed total expression of all isoforms (data not shown). Indeed, for all constructs analyzed in this report, efficient surface expression was documented by flow cytometry (data not shown).

To study the HHV-6 receptor activity of CD46 variants, a vaccinia-based reporter gene cell fusion assay (29) was previously modified for HHV-6 (6) was employed. Effector cells were generated by infecting HSB-2 cells with HHV-6 (subgroup A, strain GS); after 3–4 days, the cells were infected with a vaccinia recombinant encoding bacteriophage T7 RNA polymerase. For targets, nonhuman RK13 cells were transfected with plasmids containing the genes for the various CD46 isoforms linked to a vaccinia promoter (or with the pSc59 parental vector as a negative control); the cells were then infected with a vaccinia recombinant containing the E. coli LacZ gene linked to the T7 promoter. After a 2.5-h incubation, cell fusion was scored by measuring β-galactosidase activity in nonionic detergent cell lysates. In this and all subsequent experiments, we compared results of HHV-6 fusion with MV fusion, which also depends on CD46 as a receptor; in this case effector NIH 3T3 cells co-expressed vaccinia-encoded MV F and HA glycoproteins.
As shown in Fig. 2, HHV-6 effector cells used each of the four isoforms comparably (within a 2-fold range). These results indicate that the critical determinants on CD46 for HHV-6 receptor function do not reside in the STP B domain, and are independent of the cytoplasmic tail (1 or 2). Similar findings were obtained for MV fusion, consistent with previous reports (13, 34).

Localization of the CD46 Determinants Required for HHV-6 Receptor Function to the SCR Domains—To further delineate the CD46 determinants involved in HHV-6-mediated fusion, we examined target cells expressing chimeras between CD46 and DAF, the most closely related member of the RCA family. Fig. 3 shows that full length DAF did not function for HHV-6 fusion. By contrast, fusion activity was observed with a chimera (CD46-GPI) containing all four SCRs of CD46 plus the STP B domain, linked to a small portion of the DAF STP region and GPI anchor. The HHV-6 receptor activity of the CD46-GPI chimera was comparable to that of wild-type CD46 (compare Figs. 3, 4, and 5; also data not shown). Together with the results described above, we concluded that the C-terminal regions of CD46 (membrane-proximal B and C STP domains and the unknown region, transmembrane, and cytoplasmic tail) are not required for HHV-6 receptor activity. Rather, the critical determinants are located in the SCR domains. Consistent with previous reports (14), similar results were observed for MV fusion.

Genetic Mapping of Specific CD46 SCR Domains Required for HHV-6 Receptor Function—Next, we tested chimeras with distinct CD46 SCRs cloned into the DAF background. Fig. 3 shows that no HHV-6 fusion occurred with any of the single CD46 SCR substitutions (chimeras DM1, DM2, DM3, and DM4). The same result was obtained for MV glycoprotein-mediated fusion, consistent with previous findings (14). Interestingly, chimera DM234, containing SCR1 of DAF and SCRs 2, 3, and 4 of CD46, supported robust HHV-6 fusion, indicating that CD46 SCR1 is dispensable. However chimera DM12 containing SCRs 1 and 2 of CD46 and SCRs 3 and 4 of DAF failed to support HHV-6 fusion. The results for MV receptor activity were strikingly different; chimera DM234 was nonfunctional, whereas chimera DM12 gave potent activity. These results clearly demonstrate that distinct CD46 determinants are required for CD46 to serve as a receptor for HHV-6 versus MV: HHV-6 depends on some combination of determinants present in SCRs 2, 3, and 4, whereas MV requires SCRs 1 and 2, as previously reported (14, 15, 17–20).

To reconfirm the requirement for CD46 SCR1 and to test the need for CD46 SCR2 in HHV-6 fusion, we examined fusion mediated by two deletion mutants, one lacking SCR1 (Δ1-CD46) and another lacking SCR2 (Δ2-CD46). Consistent with the results presented in Fig. 3, HHV-6 effector cells were able to fuse with targets expressing Δ1-CD46. However, no fusion was observed with Δ2-CD46 (Fig. 4). By contrast, MV glycoproteins were unable to mediate fusion with targets expressing either deletion mutant, consistent with previous findings on the importance of SCRs 1 and 2 for MV receptor function (14). We concluded that determinants in CD46 SCR2 are required for HHV-6 receptor activity.

To further delineate the CD46 determinants for HHV-6 fu-
sion, we examined additional chimeras in which specific SCR domains of DAF were substituted for the corresponding domains in CD46 (Fig. 5). Consistent with the above results, neither HHV-6 nor MV effectors fused with targets expressing chimera x1/2-DAF, which contains SCRs 1 and 2 of DAF within the CD46 background. Moreover, HHV-6 fusion did not occur with chimera x3-DAF, in which SCR3 of DAF replaced the corresponding region of CD46; by contrast, this chimera functioned efficiently for MV fusion, consistent with the MV dependence on only SCRs 1 and 2 of CD46. These results indicate the critical importance of CD46 SCR3 for HHV-6 receptor function, in marked contrast with the requirements for MV. Neither HHV-6 nor MV fusion required CD46 SCR4, as illustrated by the results with chimera x4-DAF, in which this region was replaced by SCR4 of DAF.

Immmunochemical Mapping of Specific CD46 SCR Domains Required for HHV-6 Receptor Function—As an additional approach to examine the CD46 domains essential for HHV-6 receptor function, we examined the effects of mAbs against individual SCRs in the fusion assay (Fig. 6). Consistent with the results obtained with the genetic constructs, we observed that HHV-6 fusion with target cells expressing endogenous CD46 was inhibited by mAb M177, which is directed against SCR2 (15, 18), as well as by mAb M160 directed against SCR3 (15); by contrast, mAb GB24 against SCR4 (35, 36) did not inhibit HHV-6 fusion; in fact, enhancement was consistently noted. These results provide further support for the involvement of CD46 SCRs 2 and 3 in HHV-6 fusion. However, unexpected results were obtained with mAb Tra2.10 directed against SCR1 (18, 35). The inhibition of HHV-6 observed with this antibody (as well as with two other SCR1-directed mAbs J4.48 and E4.3, data not shown) would not have been predicted on the basis of the findings above with the genetic constructs.

The mAb sensitivity of MV fusion (Fig. 6) closely paralleled the results obtained with the genetic constructs. Thus MV fusion was significantly inhibited by the mAbs against SCR1 and SCR2 but minimally affected by the mAbs against SCR3 or SCR4; these results closely parallel published findings with mAb inhibition of MV infectivity/fusion (14–16, 18, 20), including the incomplete inhibition of MV frequently observed with Tra2.10 and other mAbs against SCR1 (15, 18, 20). As a negative control, none of the anti-CD46 mAbs showed significant inhibition of fusion between effector cells expressing HIV-1 Env and target cells expressing CD4 plus CXCR4.

DISCUSSION

The results presented herein demonstrate that SCR domains 2 and 3 of CD46 are required for HHV-6 receptor activity; this differs markedly from the requirement of SCRs 1 and 2 for MV. Our conclusions are derived from analyses of both the functionality of different CD46 variants in supporting fusion and the effects of mAbs specific for individual SCRs. For HHV-6 fusion, both approaches clearly indicate the requirement for SCRs 2 and 3 and the dispensability of SCR4. However, regarding SCR1, discrepant results were obtained by the two approaches. The molecular-genetic analyses clearly demonstrated efficient
HHV-6 receptor activity for CD46 constructs lacking SCR1, either by substitution with the corresponding region of DAF or by deletion; however, mAbs directed against SCR1 showed significant inhibition of HHV-6 fusion. A plausible explanation is that such antibodies might sterically impair the ability of the virus to interact with SCRs 2 and 3 or induce conformational changes in the relevant SCRs; alternatively, antibody binding to SCR1 might interfere with post-binding events in the fusion process. Similar proposals were offered to explain the blocking effect of a SCR3-directed mAb on MV infection (15). A related point is the prominent stimulation of HHV-6 fusion induced by the anti-SCR4 mAb. Parallel findings have been observed for antibody effects on the interactions of different enteroviruses with DAF, whereby virus binding to one SCR was greatly enhanced by a mAb directed to an alternate SCR, with corresponding increases in infectivity (37); the results were interpreted in terms of possible mAb-induced conformational changes that enhance accessibility of the relevant SCR. Finally, we note another line of evidence for our conclusions about the CD46 determinants required for HHV-6 receptor function: we have identified a specific HHV-6 glycoprotein that binds to CD46, and the patterns of co-immunoprecipitation with mAbs against different CD46 regions and with different CD46 constructs support the involvement of SCRs 2 and 3, but not SCRs 1 and 4.2

The present findings concerning CD46 domain usage can be considered in terms of various aspects of HHV-6 pathogenesis. CD4+ T lymphocytes have generally been considered to be the major targets for HHV-6 replication in vivo (1–3). However, recent studies have emphasized the importance of monocyte/macrophages during the acute stage of infection (38); such cells

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could contribute to the latency of HHV-6, as well as to the transport of the virus across the blood-brain barrier. Our present finding that all four CD46 isoforms function effectively as HHV-6 receptors suggests that differential expression of the various isoforms on different cell and tissue types is unlikely to be a major determinant of HHV-6 tropism. Also of potential significance for pathogenesis is that CD46 engagement by HHV-6 causes down-modulation of IL-12 secretion by activated blood-derived macrophages, as has been shown previously for MV in these same cells (39) and in vivo (40). Thus, this potentially immunosuppressive effect can be induced by distinct viruses that interact with different regions of CD46.

Our findings add to the growing list of pathogens that use distinct domains of CD46 as a cellular receptor for attachment and/or entry. Binding of Neisseria gonorrhoeae pili to host cells depends on determinants in the SCR3 and STP regions; the cytoplasmic tail also influences the stability of the interaction (41). The M protein of group A streptococcus promotes adhesion to keratinocytes via determinants within SCRs 3 and 4 (42). For each pathogen whose CD46-interacting regions have been defined, the determinants are distinct from (although overlap-ping with) those involved in normal ligand binding and cofactor function, i.e. CD46 SCRs 2–4 are involved in binding of both C3b and C4b; SCR1 also influences C4b binding (7, 8, 15, 35, 43).

Critical receptor domains have also been identified for pathogenic microorganisms that interact with other members of the RCA family. For example, determinants of DAF have been defined for the binding of several enteroviruses (44), including SCR1 for the Coxsackievirus A21 (45) and enterovirus 70 (46), SCRs 2 and 3 for the cardioviralent Coxsackie B3 strains (47), SCR3 for echovirus 11 (48), and SCRs 2–4 for echovirus 7 (49). The adhesins of different E. coli strains associated with urinary tract infections bind to various determinants of DAF on SCR3 or SCR4 (50, 51). Another RCA protein, complement receptor 2 (CR2 or CD21) expressed on the surface of B lymphocytes, serves as a receptor mediating infection by Epstein-Barr virus; SCRs 1 and 2 have been implicated in this interaction (52).

The x-ray crystallographic structure of a CD46 soluble fragment containing SCRs 1 and 2 has been reported and interpreted in terms of detailed mutagenesis and immunochemical studies to map the MV binding site within this region (53). In the resulting model, MV HA interacts with a large glycan-free surface extending from the top of SCR1 to the bottom of SCR2; accordingly the interacting region of the HA is also predicted to be an extended surface. It is noteworthy that the N-glycosylation site on SCR2 has been shown to be required for MV receptor function (54); the atomic structure reveals that the SCR2 glycan protrudes from the opposite side of the virus-binding surface, and may possibly interact with protein residues to stabilize the conformation of SCR2 relative to SCR1 (53).

The mode of MV/CD46 interaction differs markedly from other virus/receptor associations for which atomic structures have been obtained (e.g. HIV-1 gp120/CD4; rhinovirus capsid/ICAM-1); in each of these cases the receptor is a member of the immunoglobulin family, and only a limited region within a single domain of the receptor interacts with a recessed site on the viral protein. Another insight that has emerged from structural studies of proteins is that there is considerable flexibility between adjacent SCRs, as well as differences in interdomain orientations between adjacent SCRs of different RCA proteins (9, 53, 55). With these concepts in mind, it will be most interesting to examine an array of point mutations in SCRs 2 and 3 to more precisely define the HHV-6 interaction sites on CD46 (and also on the interacting viral glycoprotein). Such studies will likely provide new perspectives on how the RCA family provides such an extraordinary range of related structures available for exploitation by diverse pathogens.

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