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Functional and Structural Interactions between Measles Virus Hemagglutinin and CD46

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We analyzed the roles of the individual measles virus (MV) surface glycoproteins in mediating functional and structural interactions with human CD46, the primary MV receptor. On one cell population, recombinant vaccinia virus vectors were used to produce the MV hemagglutinin (H) and fusion (F) glycoproteins. As fusion partner cells, various cell types were examined, without or with human CD46 (endogenous or recombinant vaccinia virus encoded). Fusion between the two cell populations was monitored by a quantitative reporter gene activation assay and by syncytium formation. MV glycoproteins promoted fusion with primate cells but not with nonprimate cells; recombinant CD46 rendered nonprimate cells competent for MV glycoprotein-mediated fusion. Markedly different fusion specificity was observed for another morbillivirus, canine distemper virus (CDV): recombinant CDV glycoproteins promoted fusion with primate and nonprimate cells independently of CD46. Fusion by the recombinant MV and CDV glycoproteins required coexpression of H plus F in either homologous or heterologous combinations. To assess the role of H versus F in determining the CD46 dependence of MV fusion, we examined the fusion specificities of cells producing heterologous glycoprotein combinations. The specificity of H_{MV} plus F_{CDV} paralleled that observed for the homologous MV glycoproteins: fusion occurred with primate cells but not with nonprimate cells unless they produced recombinant CD46. By contrast, the specificity of H_{CDV} plus F_{MV} paralleled that for the homologous CDV glycoproteins: fusion occurred with either primate or nonprimate cells with no dependence on CD46. Thus, for both MV and CDV, fusion specificity was determined by H. In particular, the results demonstrate a functional interaction between H_{MV} and CD46. Flow cytometry and antibody coprecipitation studies provided a structural correlate to this functional interaction: CD46 formed a molecular complex with H_{MV} but not with F_{MV} or with either CDV glycoprotein. These results highlight the critical role of the H glycoprotein in determining MV specificity for CD46-positive cells.

Paramyxovirus virions are coated with surface projections that mediate virion attachment to target cells displaying the appropriate surface receptors. Fusion at neutral pH between the virion and plasma membranes then ensues, resulting in delivery of the nucleocapsid into the cytoplasm (reviewed in references [ref.] 31, 40, and 48). By a related process, cells displaying the viral glycoproteins at their surfaces can fuse with receptor-bearing cells, resulting in the formation of multinucleated giant cells (syncytia). In members of the morbillivirus genus, such as measles virus (MV) (reviewed in ref. 8) and canine distemper virus (CDV) (reviewed in ref. 6), the surface projections are composed of two integral membrane glycoproteins: the hemagglutinin (H), which mediates cell attachment, and the fusion glycoprotein (F), which promotes membrane fusion (40, 48). Recently, the human CD46 antigen (membrane cofactor protein), a type 1 integral membrane glycoprotein found on nearly all human tissues and cell types (32), was identified as the primary cellular receptor for MV. This conclusion derived from studies with a monoclonal antibody (MAb) which inhibited MV binding (42) and was subsequently shown to recognize CD46 (41). These findings, coupled with the demonstration that recombinant human CD46 renders rodent cells susceptible to MV binding, infection, and syncytium formation (13, 41), provided definitive evidence of a receptor function for CD46. Subsequent studies revealed that multiple isoforms of human CD46 (produced by alternate RNA splicing), as well as simian CD46, can serve as MV receptors (21, 30, 35, 38). The receptor functionality of a chimeric CD46 molecule with a glycosyl-phosphatidylinositol anchor indicates that all essential sequences reside within the ectodomain (56).

In the present study, we used recombinant vaccinia virus-based expression and assay systems to define the role of each MV glycoprotein in determining fusion specificity for CD46-positive cells. We also analyzed binding of the individual MV glycoproteins to CD46. The results highlight the crucial role of the MV H glycoprotein in functional and structural interactions with the CD46 molecule.

MATERIALS AND METHODS

Cells and culture conditions. All of the cell types used in this study were obtained from the American Type Culture Collection, Rockville, Md. HeLa (human cervical carcinoma) and NIH 3T3 (mouse embryo) cells were grown in DMEM-10 (Dulbecco's modified Eagle's medium [Quality Biologicals, Rockville, Md.] supplemented with 10% fetal bovine serume [Sigma, St. Louis, Mo.], 2 mM L-glutamine, and 50 μg of gentamicin [Gibco BRL, Gaithersburg, Md.] per ml). RK_{13} (rabbit kidney) and BS-C-1 (African green monkey kidney) cells were grown in MEM-10 (Eagle's minimal essential medium [Quality Biologicals] supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg of

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gentamicin per ml). Cultures were maintained in a humidified tissue culture incubator at 37°C in 5% CO_2.

Recombinant vaccinia viruses. Standard methods of homologous recombination were used to introduce foreign genes into the thymidine kinase locus of the vaccinia virus Western Reserve (WR) strain (17). All recombinants were subjected to three rounds of plaque purification, and their titers were determined on BS-C-1 cells. For individual experiments, cells were infected at a multiplicity of infection of 10 PFU per cell with each designated vaccinia virus recombinant.

The F and H glycoproteins were from the Edmonston strain of MV and the Onderstepoort strain of CDV. Recombination plasmids (described in ref. 51) contained the bacteriophage T7 promoter, followed by a DNA copy of the 5′ noncoding region of encephalomyocarditis virus RNA to enhance translation efficiency in the vaccinia virus system (18), followed by the morbillivirus glycoprotein open reading frame. The vaccinia virus recombinants and the plasmids from which they were derived are designated as follows: vT7-H_{MV}, encoding H of MV (H_{MV}), from plasmid pTM-H/MV; vT7-F_{MV}, encoding F of MV (F_{MV}), from plasmid pTM-F/MV; vT7-H_{CDV}, encoding H of CDV (H_{CDV}), from plasmid pTM-H/CDV; vT7-F_{CDV}, encoding F of CDV (F_{CDV}), from plasmid pTM-F/CDV. Vaccinia virus recombinant containing the morbillivirus glycoprotein cDNAs were identified by picking thymidine kinase-negative plaques and screening amplified samples by DNA slot blot hybridization and Western (immunoblot) analysis.

For production of recombinant human CD46, we used the cDNA encoding the MCP-BC2 isoform (GenBank accession number Y00651); nucleotide and amino acid numbering is as previously described (33). We prepared plasmids and corresponding recombinant vaccinia viruses encoding the full-length form, as well as a soluble secreted form (sCD46) representing the entire ectodomain. In each case, the cDNA was linked to a strong-early–strong-late synthetic vaccinia virus promoter (11a). A 1.5-kb cDNA fragment containing the entire CD46 coding sequence cloned into the *Eco*RI site of vector pSG5 (Stratagene, La Jolla, Calif.) was provided by M. K. Liszewski, Washington University, St. Louis, Mo. For full-length CD46, the 1.5-kb cDNA fragment was excised with *Eco*RI and cloned into *Eco*RI-digested plasmid pSC59 (11a). The resulting plasmid (pCB-48) was used to produce recombinant vaccinia virus vCB-48, encoding full-length CD46. For soluble CD46 (sCD46), a translation termination codon was inserted after the aspartic acid residue at amino acid 294 (nucleotide 1027), just prior to the predicted transmembrane domain, by using PCR technology (27). A PCR product was generated by using plasmid pCB-48 as the template, with one oligonucleotide primer (5′GACACAATTGTCTGTGACAGTAACAGTACTT G-3′) spanning the nucleotides from positions 839 to 870 and a second oligonucleotide primer (5′-CCGGCACTAGTTAATCCAAACTGTCAAGTATTCCT TC-3′) containing the translation termination codon as well as a new *Spe*I restriction endonuclease site. The resulting PCR fragment was first cloned into the pGEM-T vector system (Promega, Madison, Wis.) to generate plasmid pCB-50, and the nucleotide sequence of the cloned PCR fragment was verified. The 131-nucleotide *Sal*I-*Spe*I fragment was excised from pCB-50 and ligated into pCB-48, which had been digested with *Sal*I plus *Spe*I. The resulting plasmid, pCB-58, was used to construct recombinant vaccinia virus vCB-58, which encodes sCD46. These recombinant viruses were identified by picking thymidine kinase-negative plaques in conjunction with radioimmunoprecipitation analysis of metabolically [^{35}S]methionine-labeled lysates (full-length CD46) or culture supernatants (sCD46) from amplified plaques.

For bacteriophage T7 RNA polymerase production, two vaccinia virus recombinants were used. In vP11gene1 (1), the polymerase gene is linked to the natural late P11 vaccinia virus promoter; the advantages of using a late promoter in the cell fusion assay have already been described (45). In other experiments, we used vTF7-3 (19), which employs the natural early-late P7.5 vaccinia virus promoter.

Immunological reagents. The following antibodies against specific MV glycoproteins were employed: for H_{MV}, murine MAb 15 (ref. 24; provided by T. F. Wild, Institut Pasteur de Lyon) and rabbit polyclonal anti-H_{MV} serum 172 (provided by C. Örvell, Karolinska Institutet, Stockholm, Sweden); for F_{MV}, murine MAb 186 (reference 36; provided by T. F. Wild) and rabbit polyclonal anti-F_{MV} serum 180 (provided by C. Örvell). The following murine MAbs against specific CDV glycoproteins (ref. 47; obtained from C. Örvell) were employed: for H_{CDV}, MAb 1.347; for F_{CDV}, MAbs 3.633 and 4.985. For analysis of CD46, murine MAb J4-48 was purchased from Amac, Inc. (Westbrook, Maine). As negative controls, we employed murine MAbs D11 (16) and T4 (9), directed against the human immunodeficiency virus type 1 envelope glycoprotein.

Flow cytometry. For assay of surface-localized recombinant glycoproteins, NIH 3T3 cells were infected with the vaccinia viruses indicated in the legend to Fig. 1. At 3 h postinfection, the virus inoculum was removed and the cell monolayers were harvested by trypsinization, washed with complete medium, and incubated as suspension cultures overnight at 31°C at a density of 3×10^5 cells per ml. The cells were washed with phosphate-buffered saline (PBS)-fetal calf serum (FCS) (PBS containing 2.5% FCS), and aliquots containing 10^6 cells were incubated with the MAb indicated in the legend to Fig. 1 or control MAb T4 (2 μg of purified immunoglobulin G1 [IgG1] or a 1:100 dilution of ascites fluid in a total volume of 100 μl of PBS-FCS for 1 h at 4°C in a microcentrifuge tube). The cells were washed twice with 1 ml of PBS-FCS, stained for 30 min at 4°C with goat anti-mouse IgG-fluorescein isothiocyanate $F(ab')_2$ (Boehringer Mannheim, Indianapolis, Ind.; 1:20 dilution in PBS-FCS), and washed twice with 1 ml of PBS-FCS. Cell pellets were resuspended in 0.2 ml of PBS and fixed by addition of an equal volume of PBS containing 4% paraformaldehyde. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson).

For the sCD46 cell-binding assays, conditioned media were prepared by infecting 75-cm^2 flasks of NIH 3T3 cells with either vCB-58 or control WR vaccinia virus. At 3 h postinfection, the monolayers were washed twice with 10 ml of serum-free OPTI-MEM medium (GIBCO, Grand Island, N.Y.), overlaid with 5 ml of OPTI-MEM, and incubated overnight at 37°C. The conditioned media were harvested, clarified by centrifugation, and concentrated 10-fold in a 30-kDa exclusion Centricon microconcentrator (Amicon, Beverly, Mass.). NIH 3T3 cells producing the recombinant glycoproteins indicated in the legend to Fig. 5 were washed with PBS-FCS and resuspended at 10^7/ml in PBS-FCS. Aliquots (100 μl) of each lysate were mixed with conditioned media (50 μl), incubated for 1 h on ice, and washed. Cells were stained with anti-CD46 MAb J4-48 (2 μg in 100 μl) followed by goat anti-mouse IgG-fluorescein isothiocyanate $F(ab')_2$ and processed for flow cytometry as described above.

Metabolic labeling, immunoprecipitation, and coprecipitation. NIH 3T3 cells (in six-well tissue culture plates, 2×10^6 cells per well) were infected with the recombinant vaccinia viruses indicated in the figure legends. At 3 h postinfection, the inoculum was removed and the cell monolayers were washed once with 3 ml of methionine-free minimal essential medium and then overlaid with methionine-free minimal essential medium containing 5% dialyzed FCS and 100 μCi of [^{35}S]methionine per ml. The cultures were incubated overnight at 37°C. For analysis of cell-associated proteins, monolayers were washed with 2 ml of PBS and solubilized in 0.2 ml of lysis buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.5% (vol/vol) Triton X-100. After incubation on ice for 15 min, nuclei were removed by centrifugation. For analyses of sCD46, the supernatants of the infected and metabolically labeled cells were harvested and clarified by centrifugation. Direct immunoprecipitations of recombinant MV and CDV glycoproteins were performed by mixing in a microcentrifuge tube 10 μl of metabolically labeled cell lysate with the appropriate murine MAb ascities fluid (1 μl) or polyclonal rabbit antiserum (1 μl) in a total volume of 100 μl of PBS containing 0.1% Triton X-100. Reaction mixtures were incubated for 4 h at 4°C; when a murine MAb was used as the first antibody, 10 μg of rabbit anti-mouse IgG (Calbiochem, La Jolla, Calif.) was added and the incubation was continued for 1 h. Protein A-Sepharose beads (100 μl of a 20% suspension) were added, and the mixtures were incubated for 30 min with rocking. The Sepharose beads were centrifuged at $500 \times g$ for 5 min and washed twice with 1 ml of wash buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 0.1% Triton X-100.

For coimmunoprecipitations with CD46, an aliquot (50 μl) of unlabeled cell lysate (10^7 cells per ml of lysis buffer) from NIH 3T3 cells infected with either vCB48 or control virus (WR) was mixed with the cell lysates indicated in the legend to Fig. 6, containing metabolically labeled MV and/or CDV glycoproteins. After incubation for 4 h at 4°C, 2 μg of anti-CD46 MAb J4-48 or a control murine MAb was added and the samples were incubated for an additional 1 h at 4°C. Immunoprecipitates were collected with protein A-Sepharose beads as described above.

Immunoprecipitated proteins were eluted from the beads by heating for 5 min at 95°C in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 5% (vol/vol) 2-mercaptoethanol. Proteins were separated by SDS-PAGE on 12.5% gels. Bands were visualized by fluorography.

Cell fusion assay. Cell-cell fusion mediated by the morbillivirus glycoproteins was measured by using a recently described vaccinia virus-based reporter gene activation assay (45). The transfection and infection protocols were performed in DMEM supplemented with 2.5% fetal bovine serum. Briefly, a monolayer of NIH 3T3 cells was coinfected with vP11gene1, encoding bacteriophage T7 RNA polymerase, and the indicated viruses encoding morbillivirus glycoproteins. NIH 3T3 cells were chosen as the glycoprotein-producing cells since of the cell types examined, these were the least permissive as fusion partners (see Results); cell fusion within the glycoprotein-expressing population was thus minimized. A second monolayer of the designated cell type was transfected with plasmid pG1NT7β-gal (39a); this plasmid contains the *Escherichia coli lacZ* gene linked to the bacteriophage T7 promoter and the 5′ untranslated region of encephalomyocarditis virus. Transfections were performed with DOTAP transfection reagent (Boehringer Mannheim) in accordance with the recommended protocol as previously described (45). After 3 to 3.5 h at 37°C, the transfected cells were infected with either wild-type vaccinia virus WR or vCB-48, encoding full-length CD46. At 1.5 h postinfection, cells were detached by trypsinization, suspended to a density of 2.5×10^5/ml in DMEM-2.5 medium in 50-ml conical polypropylene centrifuge tubes (Falcon), and placed in a tissue culture incubator at 31°C. Following incubation overnight (10 to 16 h) to allow accumulation of the vaccinia virus-encoded proteins, the cells were washed once by centrifugation and suspended in DMEM-2.5 medium. To initiate cell fusion, aliquots containing 10^5 cells of each of two designated cell populations were dispensed into duplicate wells of 96-well flat-bottom microtiter tissue culture plates; cytosine arabinoside was added to a final concentration of 40 μg/ml to minimize fusion-independent β-galactosidase production. The total volume of each well was adjusted to 0.2 ml with DMEM-2.5 medium. The plates were transferred to a 37°C tissue culture incubator for 2.5 h, at the end of which time fusion was assessed.

For the quantitative reporter gene activation assay (45), Nonidet P-40 was added to each well to a final concentration of 0.5% (vol/vol). β-Galactosidase activity was quantified by colorimetric assay of aliquots of each lysate in 96-well flat-bottom microtiter plates with chlorphenol red-β-D-galactopyranoside

(Boehringer Mannheim) as the substrate. The rates of substrate hydrolysis were determined at ambient temperature by measuring the increase in A_{570} with a microplate absorbance reader (Molecular Devices, Menlo Park, Calif.). Results are expressed as nanograms of β-galactosidase per well, based on the rates obtained with parallel assays using a standard commercial preparation of *E. coli* β-galactosidase (Boehringer Mannheim). As a complementary method of analysis (45), β-galactosidase was detected in situ by fixing the cells with formaldehyde plus glutaraldehyde and staining them with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Boehringer Mannheim). Photographs were taken with an inverted tissue culture microscope with 20× phase-contrast and 10× ocular objectives; images were generated with Adobe Photoshop.

In most experiments, syncytium formation was also monitored by direct microscopic examination without staining for β-galactosidase. There was consistent agreement between the reporter gene activation assays and the syncytium analyses, although the former assays proved much more sensitive for detection of fusion events, as previously reported (45).

RESULTS

Synthesis of vaccinia virus-encoded recombinant glycoproteins. Figure 1A shows the flow cytometry analysis of cell surface-localized recombinant vaccinia virus-encoded glycoproteins of MV (left panels) and CDV (right panels). With the appropriate specific MAbs, discrete peaks were observed for cells infected with vaccinia viruses encoding MV or CDV H alone (top panels) or F alone (middle panels); in all cases, only low-level background staining was observed with a control MAb. Comparable levels of each glycoprotein were obtained when cells were coinfected with vaccinia viruses encoding both H and F of MV or CDV (bottom panels). Analysis of the polypeptides produced upon infection with these recombinant vaccinia viruses is described later.

Production of recombinant CD46 is shown in Fig. 1B. Flow cytometry (graph, left panel) was used to analyze cell surface CD46 produced upon infection with the vaccinia virus encoding full-length CD46. Anti-CD46 MAb J4-48 yielded a discrete peak, whereas only low-level background staining was observed when a control MAb was used. Additional experiments (data not shown) indicated that the amount of surface CD46 produced by the recombinant vaccinia virus system exceeded the endogenous CD46 level observed in HeLa cells by about fivefold. The autoradiogram (Fig. 1B, right panel) shows the immunoprecipitation–SDS-PAGE analysis of metabolically labeled cells infected with recombinant vaccinia viruses encoding full-length CD46 (left lane) or sCD46 (right lane). Full-length CD46 immunoprecipitated from a detergent lysate migrated as a broad band at around 67 kDa, consistent with the reported migration of this isozyme (32). The secreted sCD46 immunoprecipitated from the medium migrated as a broad band at approximately 62 kDa, consistent with the absence of the transmembrane and cytoplasmic domains (6.1 kDa).

Effect of recombinant CD46 on fusion mediated by MV glycoproteins. Figure 2 shows the ability of the recombinant MV H and F glycoproteins produced on one cell population to mediate fusion with different types of partner cells. The glycoprotein-producing cells also contained vaccinia virus-encoded T7 RNA polymerase; the partner cells contained the transfected *lacZ*-containing plasmid. Fusion was assessed both by in situ staining (photomicrographs) and by measuring β-galactosidase activity in Nonidet P-40 cell lysates (numbers in insets); similar conclusions were reached on the basis of direct microscopic analysis of syncytium formation without staining for β-galactosidase (data not shown). The left-hand panels of Fig. 2 illustrate the fusion specificity of the recombinant MV glycoproteins. Extensive fusion occurred with human HeLa (top panel) and simian BS-C-1 (data not shown) partners, as judged by the presence of large, blue-stained syncytia and high levels of β-galactosidase activity in the cell lysates. By contrast, fusion was not observed with either murine NIH 3T3 (middle panel)

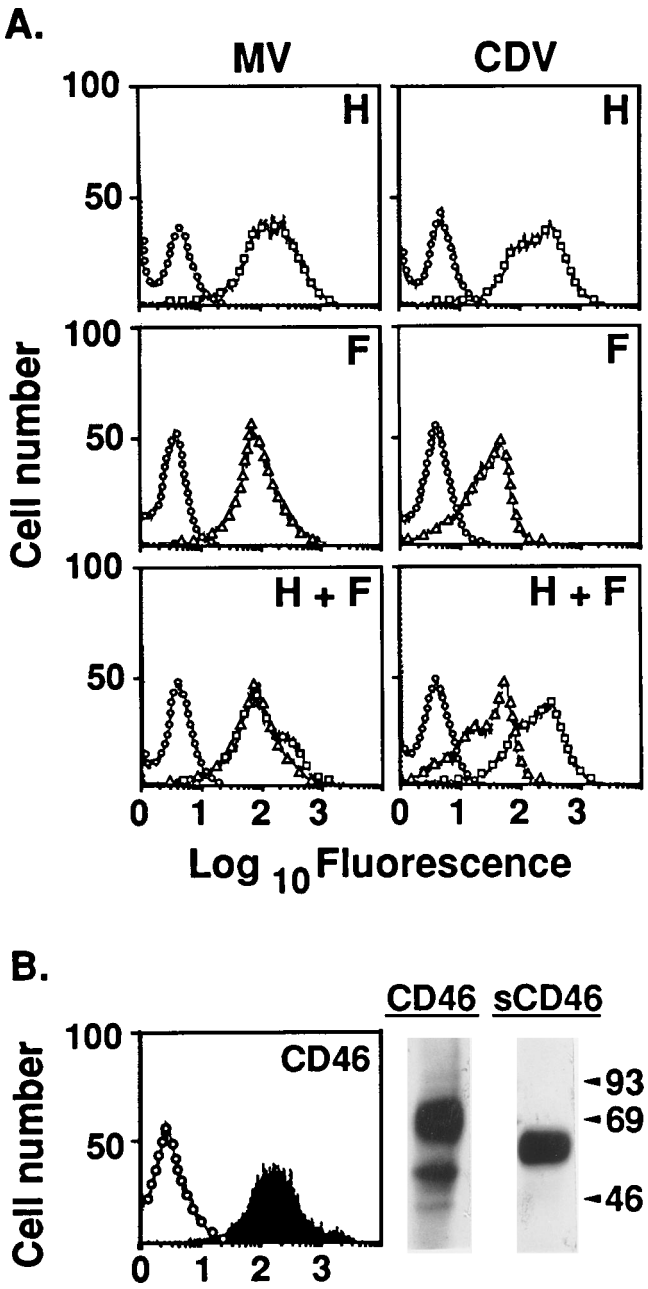

FIG. 1. Production of MV and CDV glycoproteins and CD46 with vaccinia virus vectors. (A) Flow cytometry analysis of surface-localized recombinant MV (left panels) and CDV (right panels) glycoproteins. NIH 3T3 cells were coinfected with vTF7-3, encoding T7 RNA polymerase, plus recombinant vaccinia virus vectors encoding the glycoproteins indicated (H, top panels; F, center panels; H and F, bottom panels). The following immunological reagents were employed: MAb 15 for H_{MV} (□), MAb 186 for F_{MV} (△), MAb 1.347 for H_{CDV} (□), and MAb 3.633 for F_{CDV} (△). As background controls, the same cells were incubated with MAb T4 (○). All samples were stained with goat anti-mouse IgG-fluorescein isothiocyanate and analyzed by flow cytometry. (B) Analysis of CD46. (Left panel) NIH 3T3 cells were infected with recombinant vaccinia virus vCB-48 encoding full-length CD46. Cells were incubated with anti-CD46 MAb J4-48 (filled curve) or with control MAb T4 (○) and then stained with goat anti-mouse IgG-fluorescein isothiocyanate. Samples were analyzed by flow cytometry. (Right panel) NIH 3T3 cells were infected with vCB-48 or vCB-58 encoding full-length CD46 or sCD46, respectively. After metabolic labeling, full-length CD46 was immunoprecipitated from a detergent cell lysate (left lane) and sCD46 was immunoprecipitated from the medium (right lane). The numbers on the right are molecular sizes in kilodaltons.

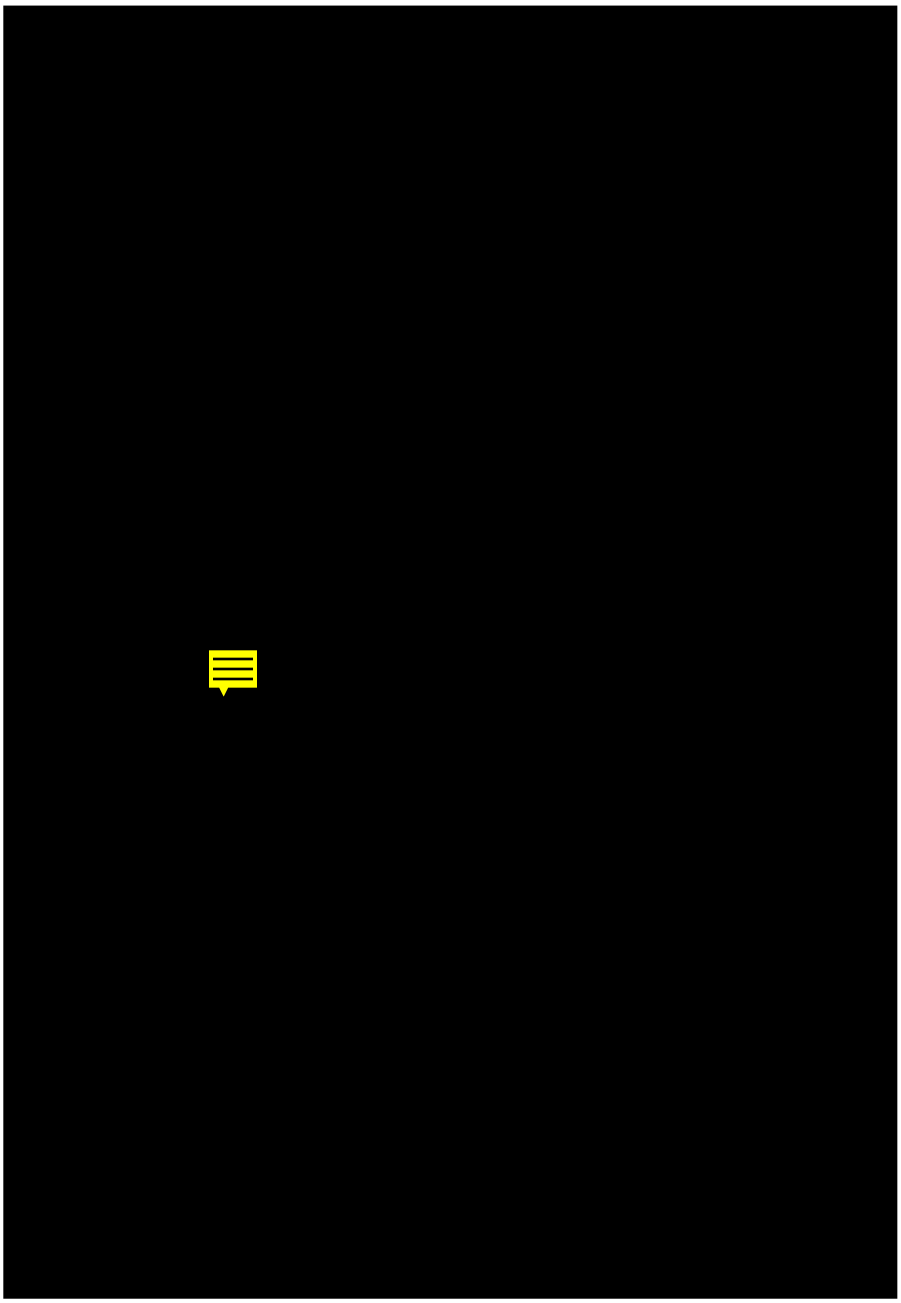

FIG. 2. Cell type specificity of MV glycoprotein-mediated fusion. One population of NIH 3T3 cells was coinfected with vaccinia virus recombinants encoding T7 RNA polymerase plus the MV F and H glycoproteins. As partner cell populations, HeLa, NIH 3T3, and RK_{13} cells were transfected with the *lacZ*-containing plasmid and infected with wild-type vaccinia virus WR (left panels). Alternatively transfected NIH 3T3 and RK_{13} cells were infected with the vaccinia virus recombinant encoding CD46 (right panels). Mixtures of the glycoprotein-producing cells and the partner cells indicated were prepared. Fusion was scored at 2.5 h by in situ staining (photomicrographs) or by colorimetric assay of detergent cell lysates (insets; results are expressed as nanograms of β-galactosidase per well ± the sample standard deviation for duplicate samples). Photomicrograph images were generated from slides with Adobe Photoshop.

or rabbit RK_{13} (bottom panel) partner cells, on the basis of the absence of blue-stained cells and detection of only background β-galactosidase activity in the lysates. We conclude that the fusion specificity of the recombinant MV glycoproteins produced and assayed by the vaccinia virus-based system was consistent with the known restriction of MV infection for human or simian cells (8).

In view of the recent reports that human CD46 functions as an MV receptor (13, 21, 35, 38, 41, 42), we tested whether vaccinia virus-encoded CD46 could confer fusion competence on nonprimate cells which were otherwise nonpermissive partners for MV glycoprotein-mediated fusion. In experiments whose results are shown in the right-hand panels of Fig. 2, the same MV glycoprotein-bearing cells were mixed with NIH 3T3 and RK_{13} partner cells containing the transfected *lacZ*-containing plasmid and infected with the recombinant vaccinia virus encoding CD46. The results indicate that recombinant CD46 rendered both nonprimate cell types permissive for MV

glycoprotein-mediated fusion, as judged by both the blue-stained cells and syncytia observed in situ and the 30- to 100-fold increase in β-galactosidase activity in the detergent cell lysates. We consistently observed that the efficiency of fusion with nonprimate cells producing recombinant CD46 was less than that observed with primate cells displaying endogenous CD46, on the basis of syncytium size and the amount of β-galactosidase activity.

Fusion mediated by F and H glycoproteins of MV and CDV. To assess the role of individual MV glycoproteins in mediating functional interaction with CD46, we performed complementation analyses between the glycoproteins of MV and the related morbillivirus CDV (see below). It was necessary first to characterize the fusion specificity of CDV glycoproteins. In contrast to the results described above for MV, CDV glycoproteins mediated efficient fusion not only with primate cells (HeLa) but also with nonprimate (NIH 3T3 and RK_{13}) cells. This broad fusion specificity for the recombinant CDV glycoproteins is consistent with the known ability of CDV to productively infect cultured cells of diverse nonprimate and primate species (6).

Complementation analysis also required characterization of the fusion activities of various combinations of MV and CDV glycoproteins (Fig. 3B). HeLa cells were chosen as fusion partners in this experiment since they are permissive for both MV and CDV glycoprotein-mediated fusion; similar results (not shown) were obtained with simian BS-C-1 cells. The upper panels in Fig. 3B compare the fusogenic activities observed with individual glycoproteins versus pairs of glycoproteins. For MV and CDV, fusion was observed when both H and F were coproduced as judged by the high β-galactosidase activities; only background β-galactosidase levels were observed with the individual glycoproteins. In the lower panels of Fig. 3B, the fusogenic activities of homologous versus heterologous mixtures of MV and CDV glycoproteins were examined; fusion was observed with each heterologous mixture. These results obtained by using the reporter gene assay to reveal functional complementation between MV and CDV glycoproteins are consistent with those of previous studies based on syncytium analysis (51).

Roles of individual MV glycoproteins in mediating fusion specificity for CD46-positive cells. The markedly different fusion specificities of MV and CDV glycoproteins, coupled with the ability of heterologous mixtures of these glycoproteins to support fusion, provided a means to assess the role of H and F in determining the CD46 dependence of MV fusion. As shown in Fig. 4, we compared the abilities of heterologous and homologous glycoprotein combinations to support fusion with different cell types. The data presented are the ratios of β-galactosidase levels obtained with pairs of partner cells chosen because they highlight the specificity of MV glycoprotein-mediated fusion. Figure 4A shows the results obtained with NIH 3T3 partner cells plus or minus recombinant CD46. Fusion mediated by the homologous MV glycoproteins was greatly stimulated by CD46, consistent with the results shown in Fig. 2; by contrast, CDV fusion was not stimulated by CD46. When the heterologous glycoprotein combinations were examined, CD46 stimulation correlated with the H rather than the F glycoprotein of MV (i.e., a high fusion ratio occurred with H_{MV} plus F_{CDV} but not with H_{CDV} plus F_{MV}). Another correlation is demonstrated in Fig. 4B, which shows the results of an experiment which examined the fusion ratio obtained with RK_{13} cells (poor partners for MV but good partners for CDV) relative to that obtained with HeLa cells (good partners for both). The nonpermissiveness of MV glycoproteins for RK_{13} cells was associated with the H rather than the F glycoprotein

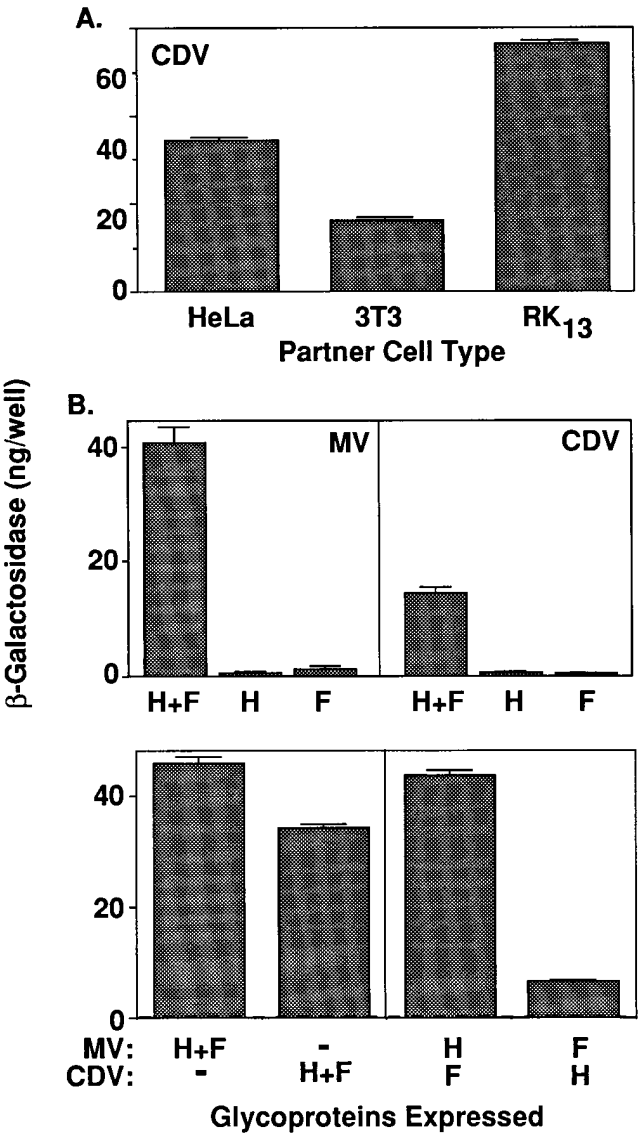

FIG. 3. Fusion mediated by MV and CDV glycoproteins. (A) Cell type specificity of CDV glycoprotein-mediated fusion. One population of NIH 3T3 cells was coinfected with vaccinia virus recombinants encoding T7 RNA polymerase plus the CDV F and H glycoproteins. As partner cell populations, HeLa, NIH 3T3, and RK_{13} cells were transfected with the *lacZ*-containing plasmid and infected with wild-type vaccinia virus WR. Cell mixtures were prepared, and fusion was scored at 2.5 h by colorimetric assay of detergent cell lysates (error bars denote sample standard deviations for duplicate samples). (B) Fusion mediated by individual MV and CDV glycoproteins versus combinations. The upper panels show requirements for coexpression of F and H. NIH 3T3 cells were coinfected with vaccinia virus recombinants encoding T7 RNA polymerase plus both (F plus H) or individual (H or F) glycoproteins of either MV or CDV, as indicated. When only one morbillivirus glycoprotein was expressed, wild-type vaccinia virus WR was used to maintain a constant multiplicity of infection. The lower panels show homologous versus heterologous glycoprotein combinations. NIH 3T3 cells were coinfected with vaccinia virus recombinants encoding T7 RNA polymerase plus homologous (left) or heterologous (right) combinations of MV and CDV glycoproteins, as indicated. As a negative control, cells expressed H_{CDV} and no other morbillivirus glycoprotein (dashed line). In both the upper and lower panels, the partner population was HeLa cells transfected with the *lacZ*-containing plasmid and infected with wild-type vaccinia virus WR. Cell mixtures were prepared, and fusion was scored at 2.5 h by colorimetric assay of detergent cell lysates (error bars denote sample standard deviations of duplicate samples).

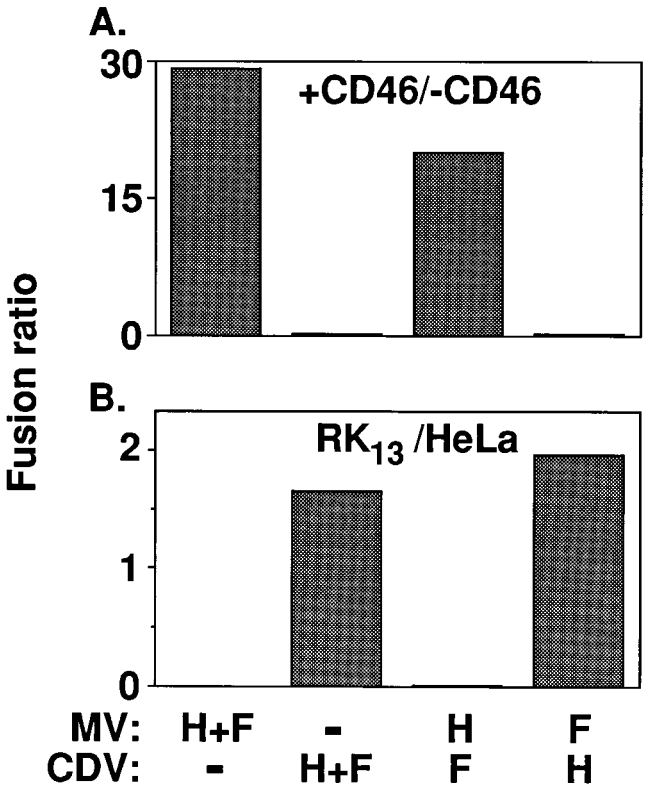

FIG. 4. Roles of individual MV and CDV glycoproteins in mediating fusion specificity. One population of NIH 3T3 cells was coinfected with vaccinia virus recombinants encoding T7 RNA polymerase plus combinations of MV and CDV glycoproteins (homologous or heterologous). The following partner cell populations were transfected with the *lacZ*-containing plasmid and infected with the vaccinia virus indicated. (A) One portion of NIH 3T3 cells was infected with the vaccinia virus encoding CD46 (+CD46), and the other portion was infected with wild-type vaccinia virus WR (−CD46). (B) RK_{13} and HeLa cells were infected with wild-type vaccinia virus WR. Cell mixtures were prepared, and fusion was scored at 2.5 h by colorimetric assay of detergent cell lysates. For each partner cell pair, the ratios of the β-galactosidase levels (fusion ratios) were calculated for the combinations of MV and CDV glycoproteins indicated.

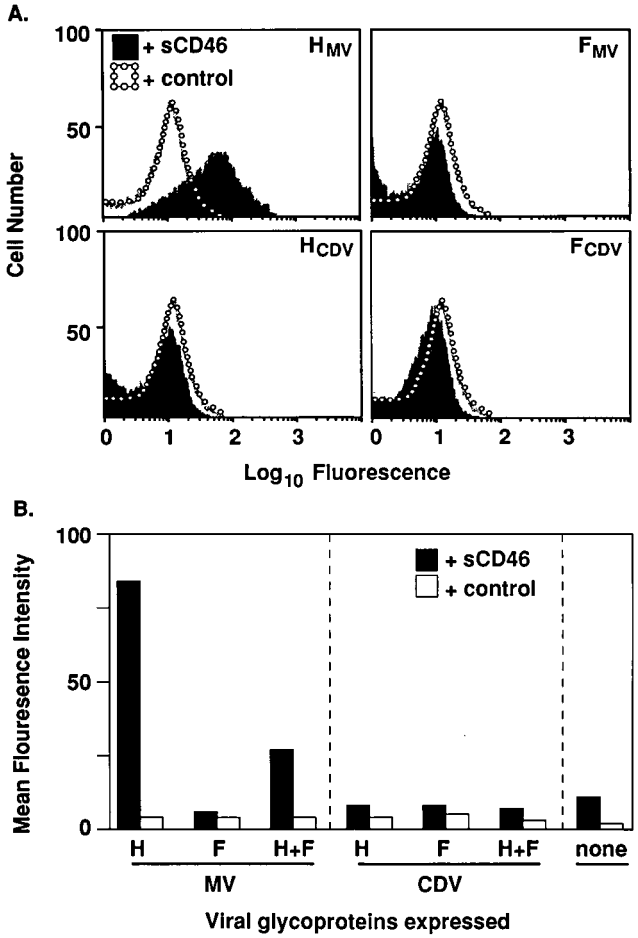

FIG. 5. Flow cytometry analysis of sCD46 binding to surface MV and CDV glycoproteins. NIH 3T3 cells were infected with vaccinia viruses encoding the MV or CDV glycoprotein(s) indicated. Cells were incubated with concentrated conditioned media from cells infected either with a vaccinia virus encoding sCD46 (+ sCD46; filled symbols) or with wild-type vaccinia virus WR (+ control; open symbols). Cells were incubated with anti-CD46 MAb J4-48 and then stained with goat anti-mouse IgG-fluorescein isothiocyanate. Panels: A, representative flow cytofluorograms; B, summary of data for cells expressing individual MV or CDV glycoproteins or combinations.

(i.e., a low RK_{13}/HeLa fusion ratio occurred with H_{MV} plus F_{CDV} but not with H_{CDV} plus F_{MV}). These results indicate that the H rather than the F glycoprotein determines MV fusion specificity; in particular, they directly demonstrate a functional interaction between CD46 and H_{MV} in the fusion process.

The H glycoprotein also appeared to determine CDV fusion specificity. In Fig. 4B, results obtained with the heterologous combinations show that CDV H rather than F enabled CDV fusion with RK_{13} cells (good RK_{13}/HeLa fusion ratios were obtained with H_{CDV} plus F_{MV} but not with H_{MV} plus F_{CDV}). Similarly, the H glycoprotein was associated with the nonresponsiveness of CDV fusion to CD46 (no CD46 stimulation with H_{CDV} plus F_{MV}, good stimulation with H_{MV} plus F_{CDV}). These findings obtained with the reporter gene assay, highlighting the critical role of H in determining CDV fusion specificity, are in agreement with previous findings based on analysis of syncytium formation when the recombinant glycoproteins were produced in various cell types (51).

Binding of CD46 to paramyxovirus glycoproteins. CD46 binding to individual MV and CDV glycoproteins was measured by two methods. The first involved flow cytometry analysis of the ability of intact cells displaying these surface glycoproteins to bind sCD46 (a genetically engineered secreted form representing the entire ectodomain). Representative patterns are shown in Fig. 5A, and the mean channel fluorescence data are summarized in Fig. 5B. The results indicate that sCD46 bound to cell surface H_{MV}, synthesized either alone or in conjunction with F_{MV}. By contrast, sCD46 binding to surface F_{MV}, or to either surface glycoprotein of CDV, was indistinguishable from the background obtained with control cells not displaying morbillivirus glycoproteins.

The second approach involved coprecipitation analysis of mixtures of detergent cell lysates (Fig. 6). Metabolically ^{35}S-labeled cells synthesizing the vaccinia virus-encoded MV or CDV glycoproteins indicated in Fig. 6 were solubilized with Triton X-100. Direct immunoprecipitation from aliquots of these labeled lysates confirmed the synthesis of each radiolabeled protein, consistent with the flow cytometry analysis presented above. Thus, direct immunoprecipitation of H_{MV} and H_{CDV} yielded predominant bands migrating at ~79 to 80 kDa, consistent with the known sizes of these glycoproteins (40, 48). For the F glycoproteins, bands were observed at the expected positions (40, 48) corresponding to the uncleaved F_0 precursors (~60 kDa) and the cleaved F_1 products (~40 kDa). Bands

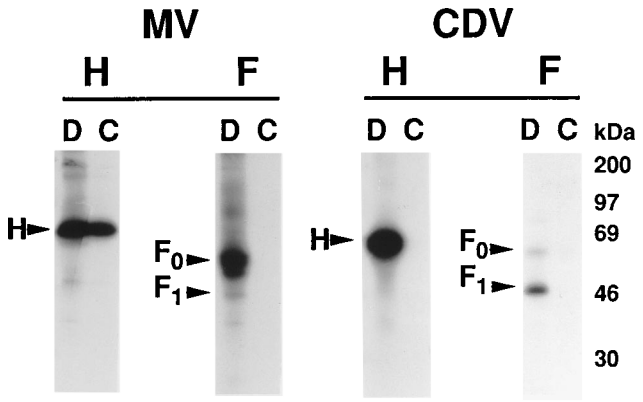

FIG. 6. Coprecipitation analysis of CD46 binding to MV and CDV glycoproteins. Triton X-100 lysates were prepared from metabolically labeled cells which had been infected with vaccinia viruses encoding the MV glycoproteins indicated. Equivalent aliquots of the lysates were analyzed by direct immunoprecipitation (D) to determine the total amount of each labeled glycoprotein and by coprecipitation (C) to determine the binding of each glycoprotein to CD46. The following antibodies were used for direct immunoprecipitation: H_{MV}, antiserum 172; F_{MV}, antiserum 180; H_{CDV}, MAb 1.347; F_{CDV}, MAb 4.985. For coprecipitation analyses, a Triton X-100 lysate from cells infected with the vaccinia virus encoding CD46 was added and anti-CD46 MAb J4-48 was used for coprecipitation.

corresponding to the F_2 products (~18 to 20 kDa) were also detected (data not shown). In the case of F_{MV}, the broadness of the F_0 band and its inefficient cleavage are consistent with reported results obtained with independently isolated recombinant vaccinia viruses (4, 60). The small amount of processed protein was evidently sufficient to support the fusogenic activity observed for this recombinant F glycoprotein.

To test the ability of CD46 to interact with the MV and CDV glycoproteins, equivalent aliquots of cell lysates containing the ^{35}S-labeled MV or CDV glycoproteins were mixed with an unlabeled cell lysate containing vaccinia virus-encoded CD46. Immunoprecipitates were obtained with MAb J4-48, which binds to CD46 but does not block MV-induced syncytium or rosette formation (13). The results indicate that the anti-CD46 MAb efficiently coprecipitated ^{35}S-labeled H_{MV}. By contrast to these positive results obtained with H_{MV}, no coprecipitation was observed when the analogous experiment was performed with ^{35}S-labeled F_{MV}. Furthermore, no coprecipitation was observed with either ^{35}S-labeled CDV glycoprotein H or F.

Additional experiments (data not shown) were performed to help interpret these results. First, the specificity of H_{MV} coprecipitation was verified by the absence of the H_{MV} band when a control isotype-matched MAb was used in place of J4-48 or when the coprecipitation was performed with a control unlabeled lysate lacking CD46 (i.e., cells infected with control vaccinia virus WR). Second, we considered the possibility that the failure to coprecipitate F_{MV} was due not simply to its inability to bind CD46 but instead to inactivation or denaturation of this glycoprotein when synthesized and solubilized in the absence of H_{MV}. This was ruled out by preparing a lysate from metabolically labeled cells synthesizing both H_{MV} and F_{MV}; addition of the CD46-containing lysate resulted in coprecipitation of H_{MV} without concomitant coprecipitation of F_{MV}. Third, we examined whether the failure of H_{CDV} to coprecipitate with CD46 was due not to absence of binding between these molecules but instead to masking of the J4-48 epitope upon binding to H_{CDV}. This unlikely explanation was ruled out by the finding that excess solubilized H_{CDV} did not interfere with the coprecipitation of H_{MV}.

Taken together, the flow cytometry and coprecipitation experiments indicate direct binding of CD46 to H_{MV}; no binding to F_{MV} was observed. Under identical conditions, binding to either glycoprotein of CDV did not occur. These results provide a structural correlate to the cell fusion experiments indicating functional interaction between CD46 and H_{MV}.

DISCUSSION

The recent discovery that human CD46 (membrane cofactor protein) serves as the primary MV receptor (13, 41) has opened important directions for elucidating the mechanism of MV entry into target cells. Experiments with MV virions have highlighted the central role of CD46 in mediating MV binding and infection (13, 21, 35, 38, 41). In the present study, we used a vaccinia virus-based expression system and a quantitative reporter gene activation assay to measure fusion between two distinct cell populations. The results directly demonstrate that recombinant CD46 serves as a fusion receptor for recombinant MV glycoproteins in the absence of other MV components, consistent with a previous analysis of syncytium formation (41).

We analyzed the relative contributions of each MV glycoprotein in the specific interactions with CD46. It is generally believed that the morbillivirus H and paramyxovirus HN glycoproteins function in the attachment of virions to target cells. In the case of MV, the major evidence derives from findings with monkey erythrocytes, including the ability of anti-H antibodies (polyclonal and monoclonal) to inhibit MV virion-induced hemagglutination (7, 20, 23, 24, 34, 39, 44, 46, 54, 55), the hemagglutinating activity of H isolated from MV virions (7, 10, 12, 20, 49), and the hemadsorption activity of recombinant cell surface H produced by using various expression systems (2, 14, 57). Regarding interaction of H with CD46, indirect evidence has been provided by the down-modulation of surface CD46 upon production of vaccinia virus-encoded H (43, 56), the CD46 dependence of purified H antigen presentation by murine B-cell transfectants (22), and the binding to CD46 of an uncharacterized MV glycoprotein fraction said to contain predominantly H (35). In this report, we directly demonstrate functional and structural interaction between H and CD46. Experiments with heterologous combinations of MV and CDV glycoproteins indicated that H_{MV} was responsible for specificity of fusion with CD46-bearing cells, thus providing clear evidence of functional interaction. Complementary results were obtained by flow cytometry and radioimmunoprecipitation studies which demonstrated specific binding between H_{MV} and CD46. No interactions between F_{MV} and CD46 were revealed in these experiments, although we cannot exclude the possibility of subtle interactions not detected by the assays employed.

We observed that for MV and CDV, production of both the H and F glycoproteins was required for fusion. This is consistent with most published results obtained with recombinant MV and CDV glycoproteins suggesting that H is required for (11, 51–53, 57, 60), or at least can stimulate (4, 5), fusion mediated by F. However, some reports have concluded that fusion can occur with F_{MV} alone (3–5), in one case under the nonphysiological condition of low pH (57). The basis for these discrepant findings is unclear, although the differences in the various methods used to produce and assay the recombinant glycoproteins presumably play a major role. It should also be noted that for the paramyxovirus genus, individual species vary in their fusion dependence on HN and F coproduction (reviewed in ref. 31; see also ref. 26 and 29).

In analyzing the MV and CDV glycoprotein coproduction requirements, we observed that fusion occurred with heterol-

ogous combinations of H of one virus and F of the other, as has been shown previously (51). A recent report, also using a vaccinia virus expression system, made the contrary suggestion that recombinant H_{MV} does not complement F_{CDV} for fusion (59). We point out that the F_{CDV} used in those experiments was encoded by a cDNA clone (58) distinct from the one used in our studies and that positive controls were not presented to document the fusogenic activity of that recombinant F_{CDV} molecule upon coproduction of homologous H_{CDV}. We therefore feel that those negative findings are outweighed by our positive fusion results obtained with F_{CDV} coexpressed with either homologous or heterologous H; however, we do not rule out the possibility that the alternative results might be due to technical differences. Our finding that fusion does not require homologous glycoprotein combinations should not be interpreted to imply the absence of specific functional interactions between H and F. In a study of recombinant glycoproteins of clinical MV isolates, efficient fusion was found to occur only with certain H-F combinations, suggesting requirements for specific functional interactions (11). Similar conclusions have been reached with the paramyxovirus HN and F glycoproteins (28, 50). Also relevant are the recent biochemical demonstrations of molecular complexes between the MV H and F glycoproteins (37) and the human parainfluenza virus HN and F glycoproteins (29). Whether the requirement for F and H coproduction reflects essential functional interactions between these molecules remains a critical question for future studies.

While studies in other laboratories have shown that transfected CD46 DNA renders nonprimate cells susceptible to MV infection (13, 21, 38), the resulting syncytia were found to be less extensive than those obtained with primate cells displaying endogenous CD46 (13). In those experiments using MV virions, suboptimal amounts of CD46 (13) or inefficiency of postentry steps in the MV replicative cycle (41) could have contributed to the results. Our studies quantitating cell fusion with recombinant proteins provide direct evidence of lower fusion efficiency of nonprimate than primate cells, despite the high-level production of CD46 achieved with the vaccinia virus vectors. It thus seems that additional factors contribute to the greater ability of primate cells to undergo MV glycoprotein-mediated fusion. In this regard, we note that surface proteins other than CD46 have been suggested to participate in MV entry into primate cells (15, 25, 61).

The cellular receptor mediating CDV infection has not been identified. Several points should be noted regarding a possible role for CD46. (i) We demonstrated that fusion mediated by CDV glycoproteins was not stimulated by recombinant CD46 on the partner cells. While this might argue against a CDV receptor function for CD46, interpretation is compromised by the fact that all of the partner cell lines tested were somewhat permissive for CDV glycoprotein-mediated fusion. It is possible that receptor levels were not limiting for fusion with these cells, thereby masking the effects of exogenous receptor production. (ii) We found that H_{CDV} (on the cell surface or in detergent lysates) did not bind to CD46 under conditions in which H_{MV} bound extensively. (iii) Others have reported that surface CD46 is down-regulated upon infection with MV but not upon infection with CDV (43). Taken together, these results suggest that a molecule(s) other than CD46 serves as the CDV receptor. Furthermore, if CD46 is involved in CDV infection, the functional determinant(s) must be present on CD46 homologs of widely divergent species, since CDV infection and CDV glycoprotein-mediated fusion both display broad specificity for nonprimate and primate cell types.

The role of the H glycoprotein in MV-CD46 interactions suggests several directions for future research. These include identification of regions of H and CD46 involved in binding, analysis of the structural determinants involved in interactions between H and F, and investigation of possible conformational changes in viral and cellular components during the fusion process.

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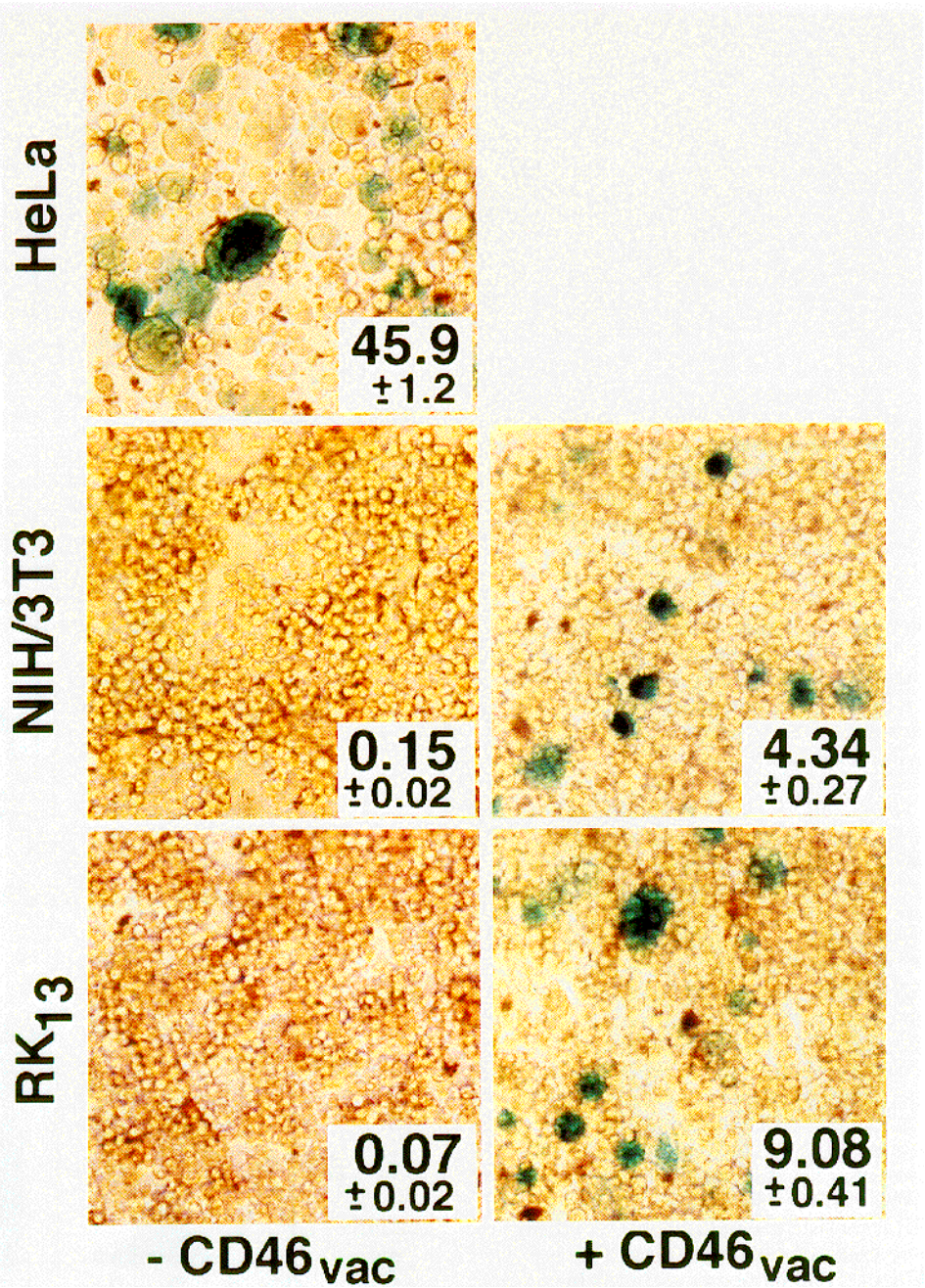
HeLa
45.9
±1.2
NIH/3T3
0.15
±0.02
4.34
±0.27
RK13
0.07
±0.02
9.08
±0.41
- CD46vac
+ CD46vac