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Artificial Mutations and Natural Variations in the CD46 Molecules from Human and Monkey Cells Define Regions Important for Measles Virus Binding

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CD46 was previously shown to be a primate-specific receptor for the Edmonston strain of measles virus. This receptor consists of four short consensus regions (SCR1 to SCR4) which normally function in complement regulation. Measles virus has recently been shown to interact with SCR1 and SCR2. In this study, receptors on different types of monkey erythrocytes were employed as "natural mutant proteins" to further define the virus binding regions of CD46. Erythrocytes from African green monkeys and rhesus macaques hemagglutinate in the presence of measles virus, while baboon erythrocytes were the least efficient of the Old World monkey cells used in these assays. Subsequent studies demonstrated that the SCR2 domain of baboon CD46 contained an Arg-to-Gln mutation at amino acid position 103 which accounted for reduced hemagglutination activity. Surprisingly, none of the New World monkey erythrocytes hemagglutinated in the presence of virus. Sequencing of cDNAs derived from the lymphocytes of these New World monkeys and analysis of their erythrocytes with SCR1-specific polyclonal antibodies indicated that the SCR1 domain was deleted in these cells. Additional experiments, which used 35 different site-specific mutations inserted into CD46, were performed to complement the preceding studies. The effects of these artificial mutations were documented with a convenient binding assay using insect cells expressing the measles virus hemagglutinin. Mutations which mimicked the change found in baboon CD46 or another which deleted the SCR2 glycosylation site reduced binding substantially. Another mutation which altered GluArg to AlaAla at positions 58 and 59, totally abolished binding. Finally, the epitopes for two monoclonal antibodies which inhibit measles virus attachment were mapped to the same regions implicated by mutagenesis.

CD46, also known as membrane cofactor protein, was recently shown to be a primate-specific receptor for the laboratory-adapted Edmonston strain of measles virus (15, 16, 22, 50). This surface glycoprotein is expressed on most types of cells and normally functions as a regulator of complement activity and helps to protect self tissue from autoimmune destruction (40, 41). CD46 is composed of four extracellular short consensus domains (SCR1, SCR2, SCR3, and SCR4) followed by a region rich in serine, threonine, and proline (called STP), a transmembrane region, and a short cytoplasmic domain at its carboxyl terminus. Variations in splicing of exons encoding STP cassettes and cytoplasmic regions yield glycoproteins which vary in size from 57 to 67 kDa and consist of all four SCR domains, a combination of STP regions, and one of two different possible carboxy termini (62, 63, 71). Each of the known splicing variants of human CD46 is capable of binding measles virus (21, 46).

The CD46 binding domains for the laboratory-adapted Edmonston strain of measles virus have recently been mapped to SCR1 and SCR2 (47) by using chimeric recombinant molecules composed of parts of CD46 and a related molecule called CD55, or decay-accelerating factor. Another group has done similar experiments with chimeras of CD46 and CD4 (11), while others expressed CD46 deletion mutants in Chinese hamster ovary cells (30) and confirmed the role of SCR1 and SCR2 in virus binding. Monoclonal antibodies specific for SCR1 and SCR2 also block measles virus binding to the host cell (30, 47). The role of carbohydrate linked to the asparagine residue in the SCR1 domain of CD46 appears to be minimal, while carbohydrate associated with asparagine in SCR2 appears to be critical for binding measles virus (42, 43).

Measles virus (25, 78) is a paramyxovirus which consists of a negative-strand RNA genome and an envelope which contains two surface glycoproteins known as hemagglutinin protein (H) and membrane fusion protein (F). A nonglycosylated membrane protein (M) lies on the inner side of the envelope and mediates an association of the glycoproteins with the nucleocapsid of the virus. H protein is responsible for virus attachment and interacts with CD46 (4, 14, 42), while F mediates penetration and syncytium formation (45, 65, 67, 70, 82). Interaction of H with its receptor has been shown to downregulate surface CD46 in cultured cells (28, 35, 49, 73–75), and four amino acids on the virus attachment protein have recently been implicated in this interaction (8). In addition, virus grown in cultured cells binds to the receptors on many, but not all, species of monkey erythrocytes to yield hemagglutination (20, 53–55, 57–59, 69). Hemagglutination assays with erythrocytes from cercopithecus and rhesus monkeys have traditionally been used to determine titers of laboratory strains of measles

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virus, indicating that the erythrocytes from many Old World monkeys do possess the receptor for this virus. However, the erythrocytes of humans, chimpanzees, and gorillas do not hemagglutinate in the presence of measles virus, which correlates with the absence of CD46 on the surfaces of erythrocytes from these higher primates (19, 52).

Since a number of species of monkey erythrocytes vary in their ability to bind measles virus and subsequently hemagglutinate (18–20), we proposed to use the receptors on these erythrocytes as "natural mutant proteins" in an attempt to further define the binding regions of CD46. In this work, we first studied the ability of New World and Old World monkey erythrocytes to hemagglutinate in the presence of measles virus. Surprisingly, none of the New World monkey erythrocytes hemagglutinated in the presence of measles virus, although it was evident that they expressed CD46 on their cell surfaces. The CD46 molecules from the lymphocytes and erythrocytes of New World monkeys all lacked the SCR1 coding region adjacent to the amino-terminal signal peptide. A specific mutation present within the CD46 molecules of baboons which could account for diminished hemagglutination activity in the presence of measles virus was noted. In addition, the effects of certain artificial mutations introduced into CD46 by site-specific mutagenesis are reported.

MATERIALS AND METHODS

Cell lines and virus. HeLa, Vero, and HuTK-143B cells were purchased from the American Type Culture Collection (Rockville, Md.). Sf9 insect cells were supplied by Invitrogen (San Diego, Calif.) and were grown in Grace's medium. HeLa, Vero, and HuTK-143B cells were propagated in Dulbecco's minimum essential medium (GIBCO-BRL, Gaithersburg, Md.) supplemented with 10% fetal calf serum. In addition, HuTK-143B cells were cultivated in the presence of 12.5 μg of bromodeoxyuridine per ml. Monkey (cercopithecus, cynomologus, and rhesus) erythrocytes were purchased from Connaught Laboratories (Toronto, Canada), and baboon erythrocytes came from Lampire Biological Laboratories (Pipersville, Pa.). Other monkey erythrocytes were obtained from the Granby Zoo (Granby, Canada), the Montreal Biodome (Montreal, Canada), the Quebec City Zoo (Quebec City, Canada), the Toronto Metropolitan Zoo (Toronto, Canada), the National Institutes of Health primate center (Poolesville, Md.), Merck-Frosst Pharmaceuticals (Montreal, Canada), and the New England Primate Center (Boston, Mass.). The Edmonston strain of measles virus was originally obtained from Erling Norrby (Karolinska Institute, Stockholm, Sweden) and was propagated in Vero monkey kidney cells as previously described (24).

CD46 polypeptide expression and production of antibodies. Polyclonal antibodies (CD46-333) directed against entire CD46 protein and antiserum directed against the SCR1 domain of CD46 protein were prepared by immunization of rabbits with purified recombinant protein. The entire CD46-coding region (isotype C2) was synthesized by PCR with oligonucleotide primers as previously described (15). The DNA fragment was cloned into the *Nhe*I site of the baculovirus expression vector BlueBac2 (pETL), and recombinant baculovirus was generated (36, 68, 80). Recombinant CD46 protein was expressed in Sf9 insect cells, resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels, electroeluted, and injected into rabbits to generate a polyclonal antibody by routine methods (66, 81). A DNA fragment containing SCR1 was synthesized by PCR with oligonucleotide primers corresponding to the 5′ end (5′-AACGGATCCT-GTGAGGAGCCACCAACA-3′) and the 3′ end (5′-TGGTGTTCGAAAATTGTCCGCAGTAGA-CTCTG-3′) of the SCR1-coding sequence. The PCR product was digested with *Bam*HI and *Hin*dIII and then cloned into the bacterial expression plasmid pT7-7His cut with the same enzymes (77). The resulting plasmid was transformed into *Escherichia coli* BL21(DE3) by electroporation, and the recombinant protein was expressed by induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The SCR1 polypeptide fused to His_6 was purified by metal chelation chromotography with Ni-nitrilotriacetic acid agarose (Qiagen) under denaturing conditions (61). Purified SCR1 polypeptide was injected into rabbits for the production of polyclonal antibodies (27). In addition, the monoclonal antibody E4.3 (specific for SCR1) was purchased from Bio/Can Scientific (Mississauga, Canada), and monoclonal antibody M75 (specific for SCR2) came from Seikugaku Inc. (Tokyo, Japan).

Hemagglutination of different primate erythrocytes by measles virus. Primate blood samples were collected from animals in Vacutainer tubes containing heparin-EDTA (Becton Dickinson, Franklin Lakes, N.J.) and stored in Alsevier's solution (GIBCO-BRL) for no more than 2 days. Cells were washed two times with phosphate-buffered saline (PBS), and a 1% (vol/vol) solution was used in the hemagglutination assays. A standard sample of concentrated measles virus (10^7 PFU/ml) was diluted serially (1:1) with PBS, and 50 μl of each dilution was added to individual wells of a 96-well microtiter plate with round-bottom chambers (Costar, Cambridge, Mass.). A 50-μl sample of the erythrocytes was added to each well. Hemagglutination was allowed to proceed for 2 h at 4°C. Erythrocytes settled to the bottom of the chambers when the assay was negative but formed a suspended matrix when the assay was positive. The assay titer was the highest dilution of virus which produced hemagglutination.

Isolation of monkey lymphocytes and synthesis of cDNAs from CD46 mRNA. Lymphocytes from different monkey species were prepared by using a Ficoll-Paque (Pharmacia, Uppsala, Sweden) discontinuous gradient. Fresh monkey blood (2 to 3 ml) was diluted with an equal volume of balanced salt solution (0.01% anhydrous D-glucose, 5 μM $CaCl_2$, 98 μM $MgCl_2$, 0.545 mM KCl, 0.126 M NaCl, and 14.5 mM Tris-HCl, pH 7.4) and layered onto 3 ml of Ficoll-Paque. The sample was centrifuged at 700 × *g* for 30 min at 20°C. The lymphocyte layer at the balanced salt solution–Ficoll-Paque interface was collected and diluted with 3 volumes of balanced salt solution. Following centrifugation at 700 × *g* for 10 min at 20°C, the lymphocyte pellet was washed once with 1 ml of balanced salt solution and then resuspended in 1 ml of TRIzol (GIBCO-BRL). Chloroform (0.2 ml) was added, and the TRIzol lysate was vortexed and left for 3 min at room temperature. The denatured extract was centrifuged at 10,000 × *g* for 10 min at 4°C. RNA in the upper aqueous layer was then precipitated with 0.5 ml of isopropanol at room temperature for 10 min and then concentrated by centrifugation at 10,000 × *g* for 10 min. Finally, the pellet was washed once with 75% ethanol, dried in the air, and resuspended in 50 μl of H_2O. Synthesis of cDNA was performed from mRNA by using the First Strand Synthesis kit (Pharmacia) and a specific CD46 primer (5′-GGGACAACACAAATTACTGC-3′). Double-stranded DNA fragments were generated by nested PCRs. Primers derived from the human CD46 sequence corresponding to 5′-GCAACTCCAACAACTATGGC-3′ and 5′-AGTGTCCCTTTCCTTCCTG-3′ were used for the first 30 rounds of PCR amplification. Interior, or nested, primers corresponding to 5′-ACAATCACAGCAATGACCCA-3′ and 5′-CGCTTTCCTGGGTTGCTTC-3′ of human CD46 were used for the following 30 rounds of PCR.

Determination of the 5′-terminal sequences of CD46 mRNA. The 5′-terminal coding regions of different monkey CD46 molecules were determined by using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, Calif.) as described in the reference manual. Poly(A)$^+$ RNA was first isolated from total RNA by using a QuickPrep mRNA purification kit (Pharmacia). Reverse transcriptase (RT) and an oligo(dT) primer were used to synthesize the first-strand cDNA from 1 μg of poly(A)$^+$ RNA. The second-strand DNA was synthesized by using a mixture of RNase H, DNA polymerase I, DNA ligase, and then T4 DNA polymerase (26). The Marathon cDNA adapter was ligated to the double-stranded DNA after synthesis of second-strand cDNA. Monkey CD46 fragment containing the 5′ coding region was generated by nested PCR with a human CD46-specific primer (5′-TAAGACACTTTGGAACTGGG-3′) and the Marathon cDNA adapter-specific primer AP1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′) for the first 30 rounds of PCR and with a human CD46-specific nested primer (5′--3′) and the Marathon cDNA adapter-specific nested primer AP2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′) for the next 30 rounds of PCR amplification. CD46 DNA fragments from the different monkeys were rendered blunt with *Pfu* polymerase (Stratagene, San Diego, Calif.) and then cloned into the *Srf*I site of the PCR Script Amp (SK+) vector (Stratagene).

SDS-polyacrylamide gel electrophoresis and immunoblot analysis. SDS-polyacrylamide gel electrophoresis and Western immunoblot analysis were performed as previously described (81). The E4.3 and M75 monoclonal antibodies (1:500 dilution) were incubated with nitrocellulose paper blotted with total proteins from OST-7 cells infected with mutant CD46 recombinant virus. Binding of monoclonal antibodies was detected by ECL chemiluminescence (Amersham, Arlington Heights, Ill.).

DNA sequencing. DNA fragments contained in PCR-Script AmP (SK(+) vectors were sequenced with an Applied Biosystems 430I automated sequencer located at the Amgen DNA sequencing facility (Amgen, Thousand Oaks, Calif.). Sequence analysis and alignments were performed with Lasergene software (DNASTAR, Madison, Wis.).

Flow cytometry analysis of monkey erythrocytes and lymphocytes. Monkey blood (50 μl) was centrifuged at 1,500 × *g* for 5 min, washed twice with 5 ml of fluorescence-activated cell sorter (FACS) buffer (PBS containing 1% bovine serum albumin, 5 mM EDTA, and 0.1% sodium azide). The erythrocytes were resuspended in 100 μl of the same buffer containing a 1:100 dilution of either preimmune antibody, CD46-333 antibody, or SCR1 antibody for 1 h on ice. Following the incubation, cells were washed twice with 3 ml of FACS buffer by centrifugation at 1,500 × *g* for 5 min. The cells were resuspended in 100 μl of the same buffer containing a 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled anti-rabbit immunoglobulin G (IgG) (heavy plus light chains) for 1 h on ice. After being washed twice with 3 ml of FACS buffer, the blood cells were suspended in 0.5 ml of FACS buffer and were subsequently analyzed on a Becton Dickinson analyzer equipped with a 15-mW argon laser at 488 nm. The data were collected and analyzed with CellQuest software.

Monkey lymphocytes were also analyzed by flow cytometry. The monkey blood samples were prepared and labeled as described above. Following the incubation

with FITC-labeled anti-rabbit IgG, the cells were washed and resuspended in 100 μl of FACS buffer. Erythrocytes were lysed by treatment of each sample with 1 ml of FACS lysis buffer (Becton Dickinson) for 1 min. The lymphocytes were washed twice with 3 ml of FACS buffer, and monkey lymphocyte fluorescence data were collected as described in the previous paragraph. Mouse OST7 cells (2×10^6 cells) which expressed mutant forms of CD46 were suspended in 1 ml of Cell Dissociation Buffer (Sigma, St. Louis, Mo.) and washed two times by centrifugation with FACS buffer. The final pellet was suspended in 100 μl of FACS buffer prior to analysis by fluorescence cytometry.

Site-specific mutagenesis of CD46 and expression of mutants by using vaccinia virus recombinants. Portions of SCR1 and SCR2 domains were replaced with point mutations or four to six alanine residues by using gel-purified oligonucleotides consisting of 30 to 40 nucleotides. Mutations were introduced into the CD46 molecule by using the QuickChange site-directed mutagenesis kit from Stratagene. The CD46-coding region (isotype C2) was cloned into PCR-Script Amp SK(+) plasmid, denatured, and annealed with two complementary oligonucleotides containing the desired mutation; the mutagenized DNA strands were extended with *Pfu* polymerase; the methylated nonmutated parental DNA template was digested with *Dpn*I; and the mutated plasmid was used to transform XL2-Blue ultracompetent cells. Mutant plasmids were isolated, and the CD46 inserts were completely sequenced. The mutagenized CD46-coding regions were excised from the PCR-Script plasmid following digestion with *Bsp*HI (5′ end) and *Bgl*II (3′ end) and ligated into the vaccinia virus vector pTM1 (containing the T7 promoter) which had been digested with the compatible restriction enzymes *Nco*I and *Bam*HI. Vaccinia virus recombinants were prepared and titers were determined by plaque assays as previously described, using HuTK-143B cells and bromodeoxyuridine selection (17). CD46 mutants were expressed in mouse OST-7 cells which contained the T7 polymerase, and protein synthesis and surface expression were monitored by Western immunoblot and FACS scan analysis.

Direct binding assays between CD46 mutants and insect cells expressing measles virus H protein. Mutant CD46-coding sequences were cloned into the pTM1 vaccinia virus expression vector, which uses the T7 promoter to direct transcription of the foreign gene. Recombinant vaccinia virus was prepared as previously described (17), and mutant CD46 molecules were expressed in mouse OST7 cells which contain the T7 polymerase. Sf9 insect cells were infected for 48 h with a recombinant baculovirus (81) which had been generated with the BlueBac2 expression vector (Invitrogen) (36, 68) and synthesized both the measles virus H protein and β-galactosidase. The insect cells were colored blue by adding Bluogal (GIBCO-BRL, Grand Island, N.Y.), at 36 h of infection, from a stock solution (50 mg/ml in dimethylformamide) to give a 0.05% (wt/vol) final concentration. Infections with recombinant vaccinia virus were allowed to proceed for 12 h; Sf9 insect cells expressing the measles H protein and β-galactosidase were incubated for 1/2 h with the vaccinia virus-infected mouse OST7 cells in the presence of PBS containing 5% fetal calf serum. Nonadsorbed insect cells were eluted by washing the mouse cells two times with PBS. Binding in 25-cm^2 tissue culture flasks could be quantitated visually under a microscope or quantitatively by the hydrolysis of *o*-nitrophenylgalactoside (ONPG) with a β-galactosidase assay kit (Stratagene). Cells were lysed in 0.5 ml of 100 mM sodium phosphate buffer (pH 7.5) containing 0.2% Triton X-100 and 1% Nonidet P-40. Aliquots of lysate (50 μl) were added to a 96-well microtitration plate, and freshly prepared buffer (100 mM sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.5) was mixed with each aliquot to give a final volume of 160 μl. A 50-μl volume of ONPG solution (4 mg/ml in 100 mM sodium phosphate, pH 7.5) was added to each well and incubated at 37°C until a yellow color was evident, after about 30 min. The reaction was terminated by addition of 90 μl of 1 M Na_2CO_3, and the color intensity was measured at 420 nm with a SpectraMax 250 enzyme-linked immunosorbent assay plate reader purchased from Molecular Devices (Sunnyvale, Calif.). Results were linear over time for an absorbance range of 0.1 to 1 A_{420} unit.

Nucleotide sequence accession numbers. The nucleotide sequences coding for the extracellular domains of monkey CD46 molecules were submitted to GenBank and have the following accession numbers: *Aotus trivirgatus*, U87914; *Papio hamadryas*, U87915; *Callimico goeldii*, U87916; *Callithrix jacchus*, U87917; *Saguinus mystax*, U87918; *Saimiri sciureus*, U87919; *Cercopithecus aethiops*, U87920; *Macaca fascicularis*, U87921; *Macaca mulatta*, U87922; and *Pithecia pithecia*, U87923.

RESULTS

Analysis of CD46 surface expression and hemagglutination of primate erythrocytes in the presence of Edmonston measles virus. Certain monkey erythrocytes are known to bind measles virus and have been routinely used to determine titers of measles virus in hemagglutination assays (19, 56). However, it is known that human and chimpanzee erythrocytes do not hemagglutinate in the presence of measles virus (19, 54). This can now be attributed to the fact that the erythrocytes of humans, gorillas, and chimpanzees do not have CD46, the receptor for measles virus, on their cell surfaces (52). African green monkey (*Cercopithecus aethiops*) erythrocytes are known to be most sensitive to measles virus-induced agglutination (18, 20, 69). We collected erythrocytes from a number of Old World and New World monkeys and assayed their ability to hemagglutinate in the presence of Edmonston measles virus which had been propagated in Vero monkey kidney cells (Table 1). The presence of CD46 on the surfaces of these monkey erythrocytes was first verified by fluorescence analysis with a polyclonal antibody specific for human CD46. All primate erythrocytes which were tested, with the exception of human and chimpanzee erythrocytes, bound the CD46 antibody. Levels of immune recognition were probably less in *Lemur macaco* due to species-specific variation of its CD46 protein. Hemagglutination assays mediated by measles virus were performed, and it was obvious that erythrocytes from South American marmosets and tamarins (*Callimico goeldii*, *Callithrix jacchus*, *Pithecia pithecia*, *Saimiri sciureus*, and *Leontopithecus rosalia*) and Madagascar lemur failed to bind measles virus (Table 1). Old World monkey erythrocytes varied in their ability to hemagglutinate in the presence of the virus. As expected, human and chimpanzee erythrocytes did not hemagglutinate, while *Cercopithecus aethiops*, *Cercopithecus diana*, *M. fascicularis*, *M. mulatta*, *Erythrocebus patas*, and *Cercocebus torquatus lunulatus* yielded high hemagglutination titers. Cercopithecus monkeys consistently yielded slightly higher hemagglutination titers than the rest of the monkeys, as previously reported (18, 20). On the other hand, erythrocytes from baboons (*Papio anubis* and *Papio hamadryas*) always produced much lower titers in

TABLE 1. CD46 FACS analysis and hemagglutination of primate erythrocytes by measles virus

Primate species	Anti-CD46 fluorescence[a]	Hemagglutination titer[b]
Homo sapiens (human)	0.359	1/1
Pan troglodytes (chimpanzee)	0.389	1/1
Cercopithecus aethiops (African green monkey)	82.14	1/64
Cercopithecus diana	81.88	1/53
Papio anubis (baboon)	68.94	1/8
Papio hamadryas (baboon)	78.25	1/8
Macaca fascicularis (cynomolgus monkey)	62.89	1/32
Macaca mulatta (rhesus monkey)	76.74	1/32
Erythrocebus patas	70.14	1/32
Cercocebus torquatus lunulatus (mangaby)	72.81	1/32
Callimico goeldii (Goeldii's marmoset)	136.8	1/2
Callithrix jacchus (common marmoset)	52.57	1/2
Pithecia pithecia (white-faced saki)	98.48	1/1
Saimiri sciureus (squirrel monkey)	38.86	1/1
Leontopithecus (lion tamarin)	47.31	1/1
Lemur macaco (black lemur)	3.90	1/1

[a] Expression of CD46 was monitored by FACS with a rabbit polyclonal antibody specific for the human cell surface antigen. The antibody-specific fluorescence intensity is represented as a number. Preimmune rabbit antibody yielded a background fluorescence of 0.200 to 0.500.

[b] Hemagglutination titers were measured in microtiter plates with standard concentrated measles virus from culture medium diluted serially by one-half in PBS buffer; an equal volume of a 1% (vol/vol) suspension of monkey erythrocytes was added to each well, and the assay mixture was allowed to incubate for 2 h at 4°C. The larger dilution indicated an increased ability of the erythrocytes to hemagglutinate in the presence of measles virus; less virus was required to cross-link erythrocytes and maintain them in suspension. Assays with Old World monkey erythrocytes were performed four times and averaged.

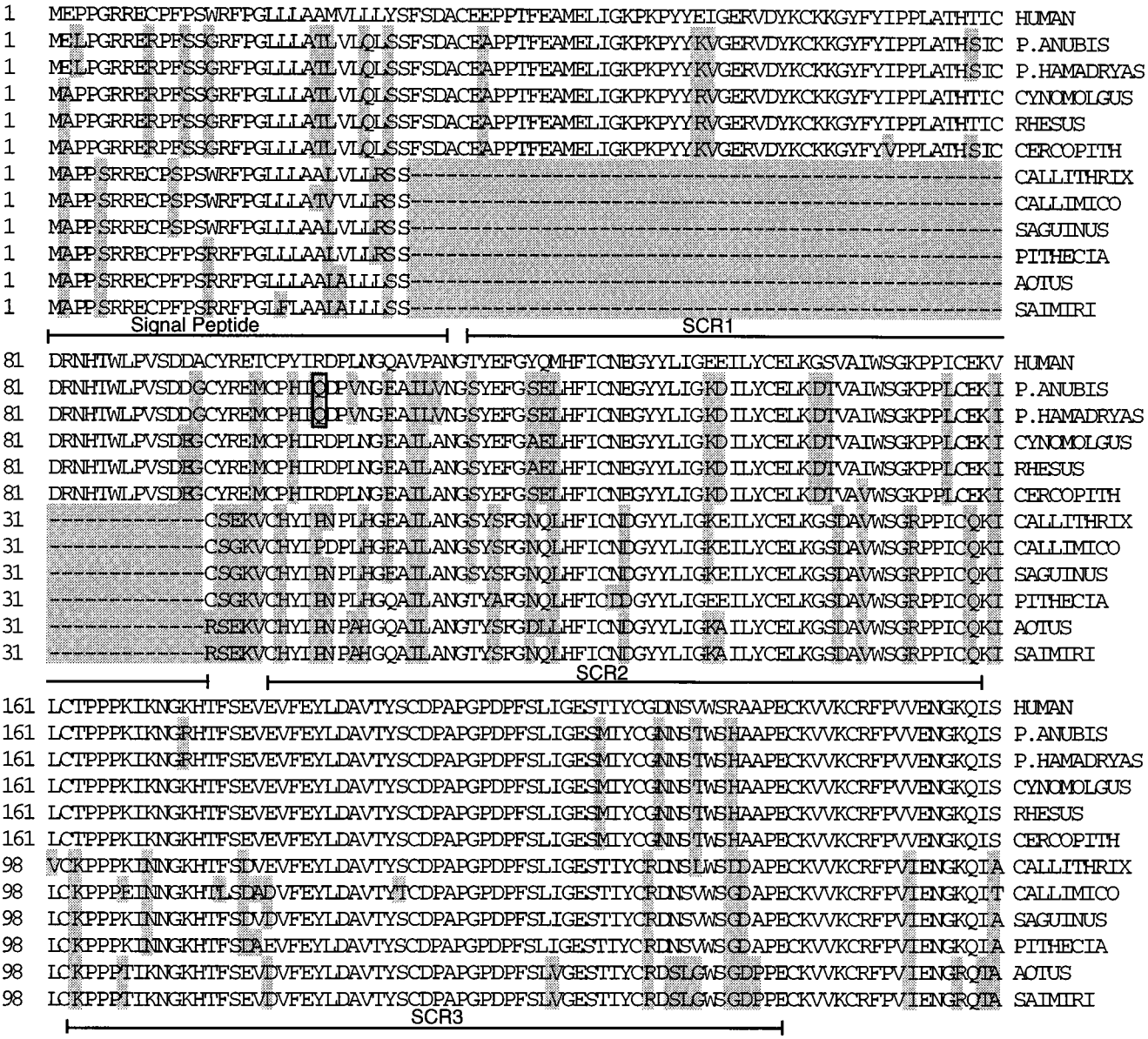

FIG. 1. Amino acid sequence alignment of signal peptides and short consensus regions from CD46 molecules found in lymphocytes of Old World and New World monkeys. Blood samples were obtained from a variety of primates originating from either Africa or South America. Lymphocytes were isolated by gradient centrifugation, total RNA was extracted, and cDNA was prepared with RT by using specific oligonucleotide primers from the conserved STP and transmembrane domains. DNA sequences were amplified by using conserved oligonucleotides upstream of the signal peptide region and regions adjacent to the SCR4 regions. The 5′ ends of New World monkeys were also cloned by rapid amplification of cDNA ends with a conserved primer from the SCR3 region. At least three independent cDNA clones were sequenced, translated to yield a predicted protein, and aligned by using the Clustal method in the Lasergene DNA analysis package. Residues which differ from the human consensus are shaded. Old World primates include humans (*Homo sapiens*), cynomolgus monkeys (*M. fascicularis*), rhesus monkeys (*M. mulatta*), baboons (*Papio anubis* and *Papio hamadryas*), and African green monkeys (*Cercopithecus aethiops*). New World primates which we tested were the common marmoset (*Callithrix jacchus*), Goeldii's marmoset (*Callimico goeldii*), the moustached marmoset (*Saguinus mystax*), the white-faced saki (*Pithecia pithecia*), the owl monkey (*A. trivirgatus*), and the squirrel monkey (*Saimiri sciureus*). The signal peptide and SCR1, SCR2, and SCR3 regions are indicated by solid lines beneath the sequences. The SCR4 domain is not shown, since it was very similar for all monkeys tested. The mutation found in baboon CD46 at amino acid 103 is highlighted by a solid box.

our assays. We proposed to isolate mRNA from the lymphocytes of these monkeys, synthesize cDNA, and sequence the regions coding for the extracellular domains of CD46 in an attempt to explain the species-specific variation in hemagglutination titers exhibited by these different erythrocytes.

Sequence comparisons of CD46 extracellular domains from different primates. Lymphocytes were isolated from the blood of different monkeys, and cDNAs were prepared by using specific primers and RT, followed by PCR (RT-PCR). The predicted amino acid sequence was deduced from at least three separate PCRs for each type of monkey. Subsequently, the signal peptide and short consensus regions (SCR1 to SCR4) were aligned by using the Clustal program from Lasergene (Fig. 1). Overall, the CD46 molecules from the different primates were highly conserved, and the protein sequences of the SCR3 and SCR4 regions from all the primates were extremely similar. The SCR3 and SCR4 domains of Old World macaques exhibited 93% identity to the human sequence,

while those of New World monkeys were 80% identical. This conservation seems consistent with the role of these regions of CD46 in complement regulation (40, 41). Since the SCR4 sequence is very similar for all monkeys, it is not shown in Fig. 1. The conserved amino-terminal signal peptides, STP regions, and membrane-spanning regions facilitated RT-PCR across the extracellular domain of CD46. It was immediately evident that the coding region for SCR1 (consisting of 63 amino acids) was missing from the cDNAs derived from the mRNAs of New World monkeys (*Callithrix jacchus*, *Pithecia pithecia*, *Callimico goeldii*, *A. trivirgatus*, and *Saimiri sciureus*). The observation that the SCR1-coding region was missing from mRNAs of lymphocytes and erythrocyte precursors of New World monkeys explains why the erythrocytes of New World monkeys did not hemagglutinate in the presence of measles virus, since this region has previously been implicated in measles virus binding (30, 47). The SCR2 region varied most from the human sequence for both Old World and New World monkeys. This region was 73% identical to the human SCR2 for Old World monkeys and 68% identical for New World monkeys. Conserved regions of identity might be expected to participate in binding to the measles virus H protein.

The CD46 sequences for different classes of Old and New World monkeys resembled each other and could clearly be used to classify the primates into different groups. Baboons, macaques, and African green monkeys belong to the family *Cercopithecidae* (51), and all have similar changes at 28 positions in the signal peptide and SCR regions when compared to the same extracellular portion of human CD46. Bands of amino acid identity at over 22 positions could easily differentiate whether the CD46 sequence belonged to an Old or New World monkey. South American monkeys could be further subclassified on the basis of 10 additional variations in the sequence shown in Fig. 1. Marmosets (*Callithrix jacchus* and *Callimico goeldii*) and tamarins (*Saguinus mystax*, *Saguinus oedipus*, and *Leontipithecus rosalia*) belong to the *Callithrichidae* family (51). On the other hand, squirrel monkeys (*Saimiri sciureus*), owl monkeys (*A. trivirgatus*), and white-faced sakis (*Pithecia pithecia*) are members of the *Cebidae* family (51). These family affiliations are reflected in amino acid variations found in the external domains of CD46 (Fig. 1).

CD46 sequences from baboons contained only seven amino acid changes when compared to the cercopithecus monkey sequence; six of these changes (positions 71, 92, 106, 113, 149, and 172) were conservative, but the other change, at position 103, produced an Arg-to-Gln substitution. This change might account for the reduced hemagglutination properties of baboon erythrocytes and was considered in subsequent studies. Cynomolgus (*M. fascicularis*) and rhesus (*M. mulatta*) monkey CD46 sequences were almost identical to the sequence of *Cercopithecus aethiops*, and all changes were minor and conservative. A slightly greater level of CD46 cell surface expression on the erythrocytes of *Cercopithecus aethiops* and *Cercopithecus diana* could explain the consistent difference in hemagglutination between cercopithecus monkeys and macaques (Table 1). It should also be noted that the Edmonston strain of measles virus has been routinely propagated in Vero and CV-1 cells, which are derived from the kidneys of an African green monkey. Propagation of this laboratory strain in culture would ultimately favor the binding of measles virus to the *Cercophithecus aethiops* CD46 receptor over those of other monkeys.

Conserved blocks of amino acids in the SCR1 and SCR2 regions could give some clues as to which regions are most important in binding to measles virus. SCR1 regions between amino acids 37 to 54 and 56 to 91 seem to be almost identical in the Old World monkeys. In addition, SCR2 regions consisting of residues 96 to 99, 103 to 109, 113 to 117, 124 to 135, 137 to 144, and 146 to 162 are almost identical in CD46 molecules from the Old World monkeys and could contribute to virus binding. Glycosylation sites at Asn83 and Asn114 are absolutely retained, 6 of 8 cysteine residues are conserved, and the 13 prolines are maintained at all positions except residues 100, 104, and 112. It was interesting that baboons, macaques, and African green monkeys contained another potential glycosylation site in their SCR3 domain at residue 213. However, this should not affect binding to H, since this region of CD46 does not appear to interact with the virus during attachment.

Polyclonal antibodies confirm that the SCR1 region is absent from CD46 molecules expressed on the erythrocytes and lymphocytes from New World monkeys. SCR1 polypeptides (amino acids 30 to 91) derived from CD46 were expressed in *E. coli*, and recombinant protein was purified and injected into rabbits in order to generate polyclonal antibodies. These antibodies were used to study surface expression of SCR1 on erythrocytes, lymphocytes, and a number of cell lines from Old and New World monkeys by fluorescent-antibody analysis. A polyclonal antibody directed against the entire CD46 molecule indicated that the erythrocytes from most Old and all New World monkeys express CD46 on their cell surface (Fig. 2). However, SCR1-specific antibodies clearly confirmed that the erythrocytes of Old World monkeys (*Cercopithecus aethiops*, *M. mulatta*, *M. fascicularis*, and *Papio anubis*) expressed the SCR1 domain of CD46 on their surface (Fig. 2A), while analysis of New World monkeys (*A. trivirgatus*, *Saimiri sciureus*, *Callithrix jacchus*, and *Saguinus oedipus*) showed that it was absent (Fig. 2B). Analysis of lymphocytes, following lysis of erythrocytes, gave identical results (data not shown). This result confirmed our RT-PCR analysis of mRNA derived from lymphocytes and explained why New World monkey erythrocytes did not hemagglutinate in the presence of the laboratory strain of Edmonston measles virus, since the SCR1 domain has been implicated in measles virus binding (30, 47).

A convenient binding assay with Sf9 insect cells expressing measles virus H protein and β-galactosidase can be used to study the binding domains of CD46. Our laboratory previously demonstrated that Sf9 insect cells infected with a recombinant baculovirus expressing the measles virus H protein (Sf9-H) were capable of binding to erythrocytes from African green monkeys (80). Since recombinant baculovirus was derived by using the BlueBac2 (pETL) expression vector (36, 68), the insect cells expressed β-galactosidase as well as measles virus H protein. We reasoned that these infected Sf9 cells grown in suspension should be capable of binding to mammalian cells with CD46 at their cell surface. Preliminary studies demonstrated that Sf9-H cells could indeed bind to HeLa cells and in addition could be stained blue in the presence of Bluogal, a substrate for β-galactosidase (29). Control Sf9 insect cells infected with wild-type *Autographa californica* nuclear polyhedrosis virus did not adhere to target cells and could be washed away. A mouse cell line containing the T7 polymerase gene (OST-7) does not normally bind measles virus. When infected with a recombinant vaccinia virus expressing human CD46 under control of the T7 promoter, these rodent cells could bind the Sf9-H cells (Fig. 3D). Mouse cells infected with wild-type vaccinia virus or a recombinant vaccinia virus expressing the SCR1, STP, and transmembrane regions of CD46 could not bind the Sf9-H cells (Fig. 3A and B). However, mouse OST-7 cells infected with a vaccinia virus recombinant expressing the SCR1, SCR2, STP, and transmembrane regions (Fig. 3C) could bind Sf9-H cells just as well as if they were infected with vaccinia virus synthesizing the whole CD46 molecule (Fig. 3D).

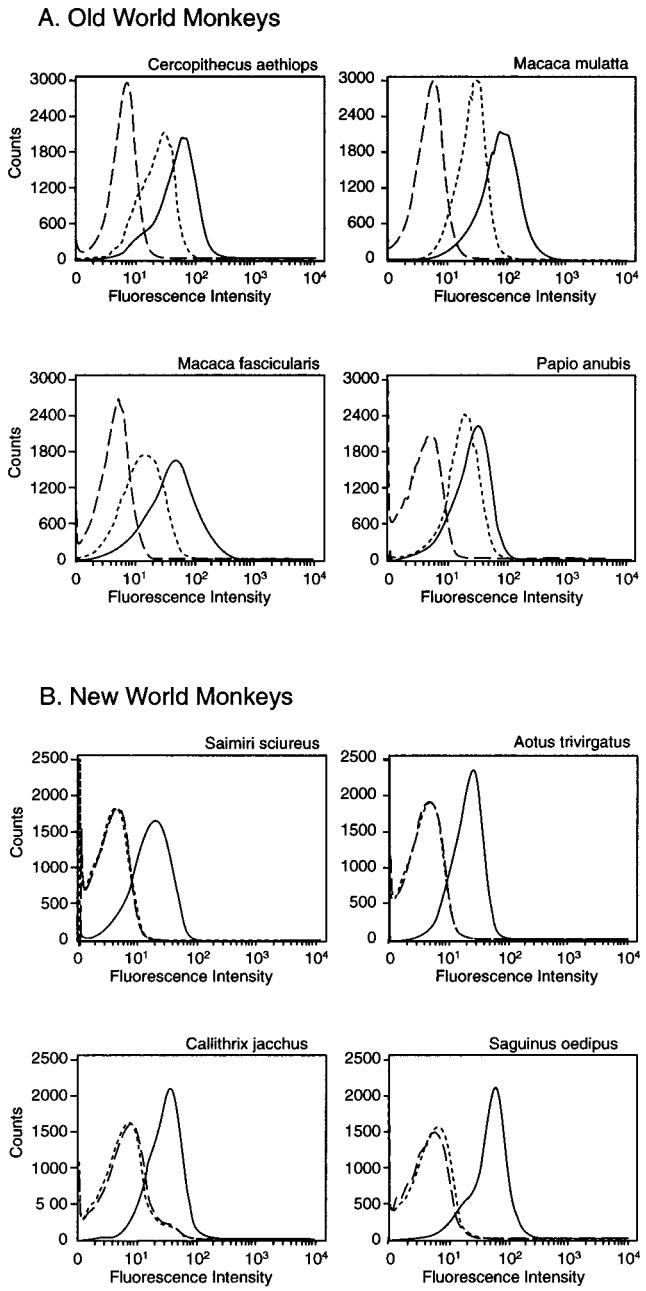

FIG. 2. Analysis of SCR1 domains and CD46 molecules from erythrocytes of Old World and New World monkeys by fluorescence cytometry. Erythrocytes from monkeys were incubated with either preimmune antiserum, SCR1-specific antiserum, or polyclonal antiserum directed against human CD46. The erythrocytes were subsequently washed twice with FACS buffer by low-speed centrifugation. Following resuspension of the pellet, the cells were incubated with FITC-labeled goat anti-rabbit IgG and again washed twice with FACS buffer by low-speed centrifugation. The labeled erythrocyte pellet was resuspended in FACS buffer and analyzed with a Becton Dickinson analyzer equipped with a 15-mW argon laser at 488 nm. The data were collected and analyzed by using CellQuest software. Cell counts are indicated on the *y* axis, and the logarithm of the fluorescence intensity is represented on the *x* axis. (A) analysis of some Old World monkey (*Cercopithecus aethiops*, *M. mulatta*, *M. fascicularis*, and *Papio anubis*) erythrocytes; (B) analysis of four different species of New World monkeys (*Saimiri sciureus*, *A. trivirgatus*, *Callithrix jacchus*, and *Saguinus oedipus*). ——, polyclonal antiserum directed against the entire human CD46 molecule; · · · · ·, polyclonal antiserum directed against SCR1; - - - -, rabbit preimmune antiserum.

These results confirm previous findings (11, 30, 47) which show that both the SCR1 and SCR2 domains of CD46 are required for measles virus binding. The results presented in Fig. 3 attest to the credibility of this assay and establish a simple direct binding assay for interaction between measles virus H protein and the human CD46 molecule. Previous investigators monitored this binding indirectly through the ability of measles virus to initiate infection in rodent cells expressing CD46 variants (47), fluorescence microscopy with measles antibodies (47), fluorescence cytometry measurements with H monoclonal antibody (30, 50), or the ability to form rosettes between African green monkey erythrocytes and the CD46-bearing target cell (15) or semiquantitatively with a biotinylated H protein overlay binding assay with nitrocellulose blots containing CD46 (42). Our new binding assay with Sf9-H cells offers the advantages of being quick, sensitive, and easy to quantitate with either Bluogal or ONPG substrates.

Site-specific mutagenesis can be used to map regions of CD46 important for interaction of the H protein. Binding of measles virus to African green monkey erythrocytes has previously been reported to be reversed by incubation with 1 M arginine, and consequently, electrostatic interactions appeared to be important in virus attachment (37). With this result in mind, we decided to initiate our mutagenesis studies by systematically replacing charged amino acids in the SCR1 and SCR2 domains with alanine. Larger stretches of the CD46 binding domains were also replaced with tracts of four to six alanine residues. Mutant CD46 molecules were expressed in mouse OST-7 cells by using recombinant vaccinia virus, and binding assays were performed with Sf9-H cells. Expression of all CD46 mutants on the surface of OST-7 cells was analyzed by fluorescence cytometry, and similar amounts of recombinant protein were expressed on all cells. Levels of mutant CD46 surface expression are presented in Table 2. Binding of Sf9-H cells to OST-7 cells expressing mutant CD46 molecules was measured colorimetrically with an ONPG assay, and the results relative to those for normal CD46 are presented in Fig. 4.

Substitution of charged amino acids at the SCR1 amino terminus (EE36/37AA and E45A) appeared to moderately affect binding and led to 25 to 30% inhibition of binding. Mutation of the glycosylation site in SCR1 at Asn83 had no effect on binding, nor did mutations of the charged residues surrounding this site. However, the mutation ER58/59AA dramatically inhibited binding, up to 80%. Mutation of RD103/104AA and another N-glycosylation site (N114A) in the SCR2 domain caused moderate but reproducible inhibition of binding, 25 and 50%, respectively. As previously stated, the Arg residue at position 103 has been replaced by a Gln in baboons (*Papio anubis* and *Papio hamadryas*), and we and others have noted that baboon erythrocytes are not nearly as effective in hemagglutination assays mediated by measles virus. Changes at amino acid 103 appear to diminish measles virus binding but not abolish it. Another laboratory also recently reported that the mutation at Asn114 destroyed this glycosylation site and reduced infections and binding by measles virus (44). It has not been shown whether this carbohydrate chain participates directly or indirectly in binding. For the most part, single amino acid mutations do not completely abolish binding of mutant forms of CD46 to Sf9-H cells. This implies that several distinct regions in SCR1 and SCR2 cooperate in binding in a conformational rather than a linear manner and that no one region of SCR1 and SCR2 is critical for virus interaction.

We were surprised to observe that multiple substitutions of four to six alanine residues at seven locations over the SCR1 and SCR2 domains (E42 to G48, K49 to E55, E58 to C64, Y70

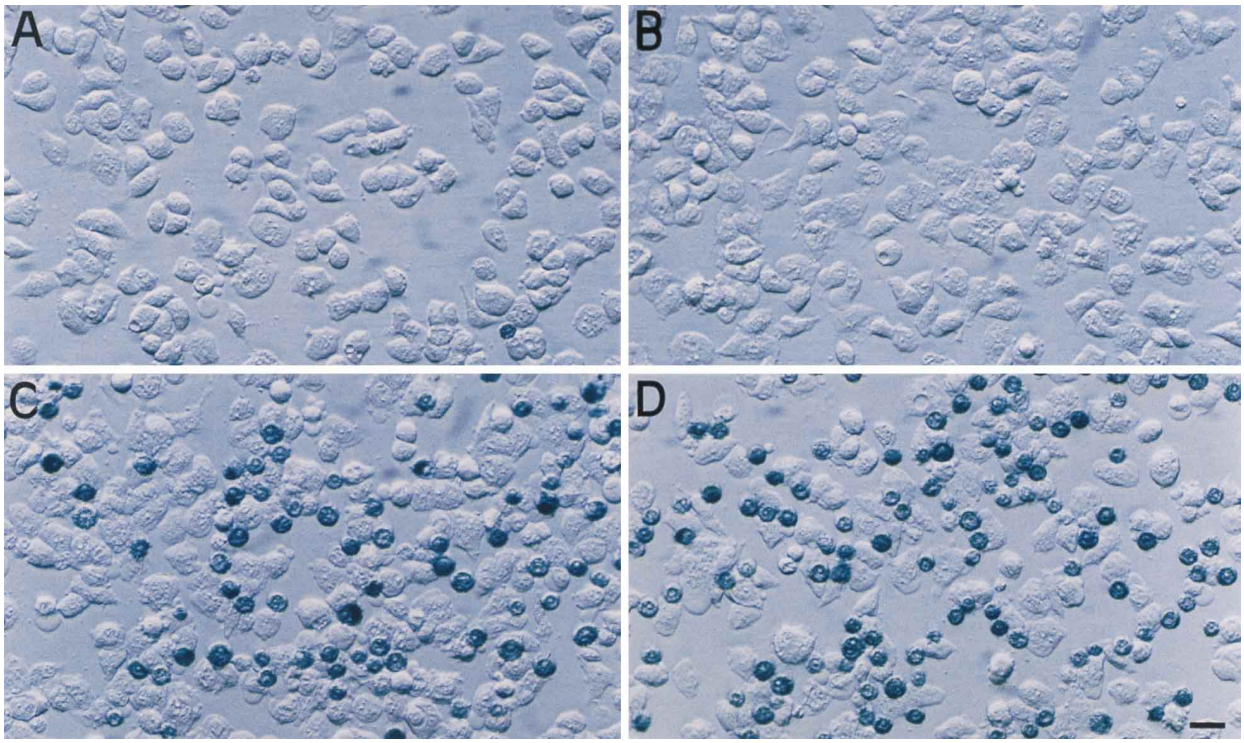

FIG. 3. Binding assay for mutant CD46 molecules by using insect cells expressing the H protein of measles virus. Mouse OST-7 cells were infected with wild-type vaccinia virus (A), vaccinia virus expressing the SCR1 domain, the STP region, and the CD46 transmembrane region (B), a recombinant vaccinia virus expressing SCR1, SCR2, the STP domain, and the CD46 transmembrane domain (C), or a recombinant virus expressing the entire human CD46 molecule (D). OST-7 cells alone normally do not bind measles virus. Sf9 insect cells infected with a baculovirus expressing measles H protein and β-galactosidase were incubated with Bluogal substrate, washed with PBS, and added to the vaccinia virus-infected cells. Binding of blue-colored insect cells to mouse cells which expressed either SCR1 and SCR2 (C) or the entire human CD46 molecule (D) was apparent.

to A75, D81 to W86, N128 to L133, and I149 to K153) all totally abolished binding to Sf9-H cells. However, substitution of residues in the hinge region between the SCR1 and SCR2 domains (Y95 to T98) had little inhibitory effect. These changes seem to indicate that major distortions of the SCR1 and SCR2 domains cannot be tolerated in binding assays. Again, the multiple substitutions did not affect display of the mutant CD46 molecules on the surface of the mouse OST-7 cells (Table 2). It is quite possible that multiple alanine mutations might disrupt intramolecular disulfide bond formation and alter the native conformation of the virus binding domains.

Monoclonal antibodies E4.3 and M75 inhibit binding of H protein by interacting with specific regions in SCR1 and SCR2. Two monoclonal antibodies which react with CD46 were previously reported to block measles virus infections and prevent attachment of the measles virus H protein to the target cell (15, 30). We were able to map the regions on CD46 which interact with monoclonal antibodies E4.3 and M75 by using recombinant vaccinia virus expressing different CD46 mutants (Table 2) and to show that they inhibited binding of Sf9-H cells (Fig. 4). E4.3 inhibited binding of normal CD46 to Sf9-H cells by 45%, while M75 yielded 67% inhibition. In order to map the monoclonal antibody epitopes, OST-7 cells were infected with vaccinia virus recombinants, and infected cell lysates were analyzed by immunoblotting with the two monoclonal antibodies; diminished reactivity of the monoclonal antibodies with mutant CD46 molecules was noted (Table 2). E4.3 antibodies appeared to recognize residues 36 to 59 in the amino-terminal domain of SCR1, while M75 recognized amino acids 103 to 114. These epitopes coincided with the regions where site-specific mutations had the most effect on virus binding. It will be interesting to note the inhibitory effects of additional monoclonal antibodies which recognize other regions of SCR1 and SCR2, when they become available.

DISCUSSION

CD46 was previously shown to be the receptor for the Edmonston strain of measles virus (15, 50). This paper expands upon the results from two other laboratories (30, 47), which claimed that the presence of both the SCR1 and SCR2 domains of CD46 is sufficient for virus binding. We proved that this process occurs via direct interaction of the H protein with SCR1 and SCR2 by using a convenient binding assay with Sf9 insect cells expressing the viral surface protein, H. In our study we were surprised to note that all New World monkeys which were analyzed did not express the SCR1 domain in CD46 molecules present on their erythrocytes and lymphocytes. This observation accounted for their inability to bind measles virus and hemagglutinate. The SCR1 deletion corresponds to a missing second exon which is not retained in the CD46 mRNA during the processing of precursor RNA; however, the exon still appears to be present in chromosomal DNA (29), which implies some irregularity in the splicing process. The compositions of the STP and cytoplasmic domains of CD46 are known to vary through a process of alternate splicing, but exons corresponding to the SCR domains have never been reported to be deleted through mRNA processing (62, 63, 71). No functional role has yet been assigned to the SCR1 domain

TABLE 2. Site-specific mutagenesis, cell surface expression, and reactivity of CD46 mutant proteins with specific antibodies

Mutation[a]	Surface expression[b] (mean fluorescence)	Reactivity with monoclonal antibody[c]:	
		E4.3	M75
CD46 C2	87.77	+++	+++
EE36/37AA	81.40	−	+++
E42A	91.50	+	+++
E45A	84.45	+	+++
KK49/51AA	87.74	−	+++
E55A	94.90	+	+++
ER58/59AA	98.80	+	+++
DK61/63AA	85.64	+++	+++
KK65/66AA	83.13	+++	+++
H77A	81.40	+++	+++
DR81/82AA	92.15	+++	+++
N83A	78.94	+++	+++
H84A	97.87	+++	+++
DD91/92AA	87.37	+++	+++
RD103/104AA	86.77	+++	−
N114A	89.95	+++	−
E118A	77.57	+++	+
H124A	101.11	+++	+++
E129A	99.50	+++	+++
E136A	78.25	+++	+++
E137A	89.01	+++	+++
E142A	104.05	+++	+++
E144A	84.99	+++	+++
K153A	97.89	+++	+++
Multiple 1 (E42–G48)	70.00	−	+++
Multiple 2 (K49–E55)	102.16	−	+++
Multiple 3 (E58–C64)	89.78	−	+++
Multiple 4 (Y70–A75)	75.57	+++	+++
Multiple 5 (D81–W86)	70.02	+++	+++
Multiple 6 (Y95–T98)	85.61	+++	+++
Multiple 7 (N128–L133)	114.33	+++	+++
Multiple 8 (I149–K153)	93.58	+++	+++

[a] The CD46 molecule (isotype C2) was subjected to site-specific mutagenesis as described in Materials and Methods. The position of the mutation is indicated by the amino acid residue numbered from the amino terminus of the polypeptide, and includes the signal peptide prior to posttranslational cleavage. The letter to the left of the number indicates the original amino acid, while the letter to the right indicates the change. Multiple mutants 1 to 8 consist of alanine tract substitutions between the indicated positions.

[b] The mean fluorescence of CD46 mutant proteins which were expressed on the surface of mouse OST-7 cells was determined by FACS analysis with a rabbit polyclonal antibody directed against CD46. Analysis was performed three times with an error of ±15%. The antibody-specific fluorescence intensity is represented as a number. Preimmune rabbit antibody yielded a background fluorescence of 0.200 to 0.500.

[c] Reactivity of the mutant CD46 molecules with monoclonal antibodies E4.3 and M75 was determined by immunoblot analysis of proteins from a mutant CD46 recombinant vaccinia virus-infected cell lysate. −, no band appeared on the blot; +, presence of a faint band; +++, indicates the monoclonal antibody recognized the mutant CD46 protein.

of this molecule, since complement components C3b and C4b have previously been demonstrated to interact with SCR2, SCR3, and SCR4 (1, 12, 30). A role in signal transduction has recently been proposed for CD46, and it was shown that interaction of this receptor with measles virus or monoclonal antibodies specific for SCR1 could downregulate the production of interleukin-12 (32). This receptor interaction could partially account for the immunosuppressive effects of measles virus. It remains to be determined if other cellular factors besides measles virus interact with the SCR1 domain. The deletion of SCR1 in most tissues of New World monkeys (29) may have interesting evolutionary implications in terms of disease and immune regulation.

Erythrocytes from the baboon *Papio cynocephalus* have previously been reported to hemagglutinate in the presence of laboratory strains of measles virus (57, 59). However, researchers showed that many Old World monkey erythrocytes, including those from *Papio cynocephalus*, *M. fascicularis*, *M. mulatta*, and *E. patas*, were less efficient in these assays than erythrocytes from the African green monkey (*Cercopithecus aethiops*) (18–20). We confirmed this result with two other species of baboon (*Papio anubis* and *Papio hamadryas*) and found that this decreased ability to hemagglutinate corresponded to a change at amino acid residue 103, which implicates this region of CD46 in measles virus binding. We were able to imitate this natural change through site-specific mutagenesis, and our experiments revealed decreased affinity for the mutant CD46 in an Sf9-H binding assay. The nearby carbohydrate attachment site at Asn114 was also shown to be important for optimal binding, since mutation of this residue to Ala also reduced interaction with Sf9-H cells. In addition, the epitope for a monoclonal antibody, M75, was mapped to the same region (amino acids 103 to 118) by using immunoblot analysis of our mutant CD46 molecules, and it was also shown to inhibit binding effectively. These preceding studies appear to implicate the region of CD46 consisting of amino acids 103 to 118 as at least one of the sites involved in virus attachment.

The artificial mutation in CD46 which changed GluArg at positions 58 and 59 to AlaAla had a dramatic effect on Sf9-H cell binding. This is a region which could be involved in electrostatic interaction with the binding region of measles virus H protein. Arginine at concentrations of 1 M has previously been shown to abrogate virus binding and hemagglutination (37). The region at amino acid 58 or 59 could contribute to salt-dependent or charged residue interaction during the attachment process (23, 37, 76). The monoclonal antibody E4.3 has previously been studied in virus attachment experiments (30, 47). There is some discrepancy between the results from the two laboratories as to the effectiveness of this antibody in inhibiting the binding of H protein to target cells, which could be related to the concentration of the reagent used in their experiments. Our results support those of Manchester et al. (47) and confirm that E4.3 was indeed a potent inhibitor of measles virus H protein attachment. We further demonstrated that the epitope for monoclonal antibody E4.3 mapped to a region of CD46 between amino acids 37 and 59 by using immunoblot analysis of our mutant CD46 molecules. Taken together, these data indicate that the region of CD46 consisting of amino acids 37 to 59 also participates in binding to the measles virus H protein.

The CD4 binding region (amino acids 39 to 59) which interacts with human immunodeficiency virus glycoprotein (gp120) has previously been mapped by other investigators through a series of site-specific mutations and interactions with monoclonal antibodies (5, 6, 9, 10, 13, 31, 48, 60, 72, 79). Synthetic peptides were also employed by other laboratories in attempts to map binding regions on CD4 but led to contradictory results (31, 38, 39, 64, 83). Originally we also attempted to perform peptide studies with measles virus and CD46 by using 25-amino-acid peptides derived from all regions of SCR1 and SCR2. Peptides at concentrations as high as 200 μM had no effect in Sf9-H binding and measles virus infectivity assays. We presumed that binding of measles virus depended on conformational epitopes rather than linear peptide epitopes. Subsequently, a genetic approach using site-specific mutagenesis was adopted in our laboratory to map binding regions on CD46. Initially we found that large mutagenic changes involving mul-

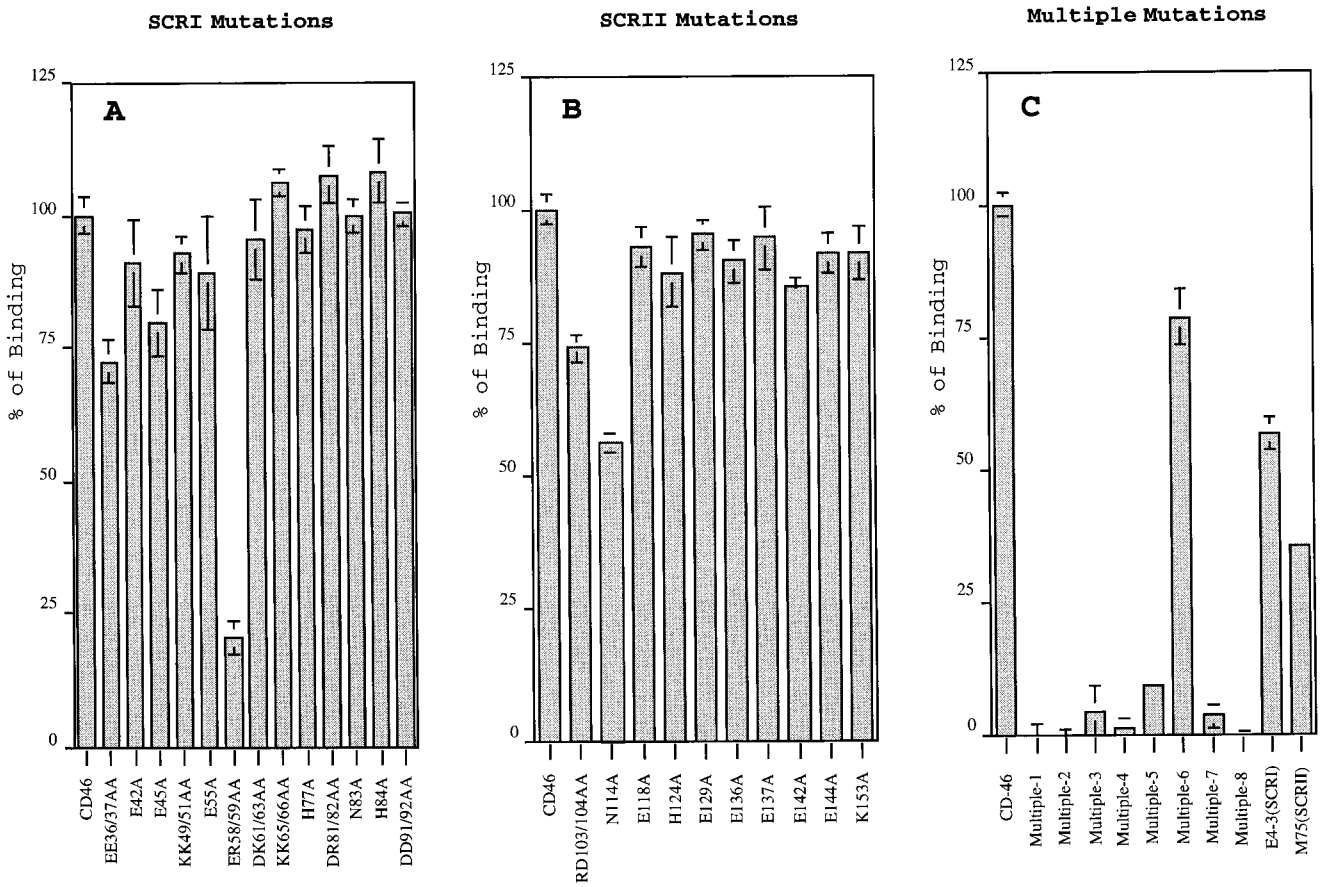

FIG. 4. Effect of CD46 site-specific mutations and monoclonal antibodies directed against SCR1 and SCR2 on binding to insect cells expressing the measles virus H protein. Mouse OST-7 cells were infected with recombinant vaccinia virus expressing the mutant CD46 molecules described in Table 2. Sf9 insect cells which expressed both measles virus H protein and β-galactosidase were added to the mouse cells for 1/2 h and subsequently washed with PBS. Binding was quantitated by a colorimetric assay for β-galactosidase with ONPG as a substrate and was compared to a standard value produced by cells expressing the normal CD46 molecule. This binding relative to that for normal human CD46 is represented on the *y* axis of the histogram, while the various mutations are listed on the *x* axis. OST-7 cells infected with wild-type vaccinia virus served as a negative control and exhibited no binding to Sf9-H cells. (A) Mutations introduced in SCR1; (B) mutations placed in SCR2; (C) multiple alanine substitutions in both SCR1 and SCR2. The inhibitory effects of monoclonal antibodies directed against SCR1 (E4.3) and SCR2 (M75) are also shown in panel C. These two commercial antibodies were diluted 1:20 and added to binding assays, and inhibition was noted. Results are expressed as means and standard deviations.

tiple alanine substitutions could not be tolerated. These multiple mutations appeared to perturb binding of Sf9-H cells to CD46, no matter which region was targeted. Surface expression of the mutated CD46 molecule was not affected, but intramolecular disulfide bond formation may have been altered. The only area where multiple alanine mutations did not dramatically affect binding was the hinge region between SCR1 and SCR2, indicating that this region was relatively refractile to mutagenesis and probably was not important in measles virus binding. Smaller point mutations were subsequently utilized in our experiments to map regions in SCR1 and SCR2 which are important for measles virus binding.

A molecular understanding of the exact interaction of measles virus H protein with CD46 will ultimately depend upon structural studies using nuclear magnetic resonance or X-ray crystallography to dissect the binding regions of H, SCR1, and SCR2. Serum factor H, like CD46, is another complement binding protein which is composed of 20 short consensus regions. The SCR15 and SCR16 regions of factor H, whose three-dimensional structure has been solved (7), has some homology to the two terminal domains of CD46. The polypeptide backbone for CD46-SCR1 and CD46-SCR2 can be crudely modeled and superimposed on that for the SCR15 and SCR16 domains from factor H. However, one can only approximate regions of interaction between the receptor and viral glycoprotein at this time. The fine structure of the intramolecular loops defined by the cysteine residues and the orientation of the SCR domains about the hinge region between SCR1 and SCR2 will likely determine the residues available in the binding site. Mutagenesis offers some clues as to which residues are important in virus binding, but these experiments are actually a lead into structural studies.

Based on the deletion of SCR1 regions in New World monkeys, we might predict that marmosets and tamarins from South America are resistant to infections by the Edmonston strain of measles virus. Studies in our laboratory indicate that owl monkey kidney cells (OMK) and marmoset kidney cells (NZP-60) also contain this deletion and cannot be infected with measles virus in the laboratory (29). However, we and others (33) have infected a marmoset B-cell line which has been immortalized with Epstein-Barr virus (B95-8), and results indicate that these cells contain both the SCR1-deleted and nondeleted forms of CD46 (29). Other researchers have reported measles virus infections in cynomolgus monkeys, mar-

mosets, tamarins, and squirrel monkeys (2, 3, 34). The disease in moustached marmosets has been reported to be characterized by severe gastroenterocolitis and immunosuppression (2), while the symptoms presented by squirrel monkeys are similar to those in humans and consist of a rash, Koplik's spots, and infection of lymphatic tissue (34). The receptor distribution in these animals is currently under study in our laboratory, and the nature of the SCR1 deletion in different organs of monkeys may determine the tissue tropism and nature of the disease in these animals. Since the SCR1 measles virus binding domain appears to be missing in these infected New World monkeys, it will be interesting to determine if another receptor besides CD46 can be used by measles virus during the process of attachment.

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REFERENCES

1. **Adams, E. M., M. C. Brown, M. Nunge, M. Krych, and J. P. Atkinson.** 1991. Contribution of the repeating domains of membrane cofactor protein (MCP; CD46) of the complement system to ligand binding and cofactor activity. J. Immunol. **147:**3005–3011.
2. **Albrecht, P., D. Lorenz, M. J. Klutch, J. H. Vickers, and F. A. Ennis.** 1980. Fatal measles infection in marmosets: pathogenesis and prophylaxis. Infect. Immun. **27:**969–978.
3. **Albrecht, P., D. Lorenz, and M. J. Klutch.** 1981. Encephalitogenicity of measles virus in marmosets. Infect. Immun. **34:**581–587.
4. **Alkhatib, G., and D. Briedis.** 1986. The predicted primary structure of measles virus hemagglutinin. Virology **150:**479–490.
5. **Arthos, J., K. C. Deen, M. A. Chaikin, J. A. Fornwald, G. Sathe, Q. J. Sattentau, P. R. Clapham, R. A. Weiss, J. S. McDougal, C. Pietropaolo, R. Axel, A. Truneh, P. J. Maddon, and R. W. Sweet.** 1989. Identification of the residue in human CD4 critical for the binding of HIV. Cell **57:**469–481.
6. **Ashkenazi, A., L. G. Presta, S. A. Marsters, T. R. Camerato, K. A. Rosenthal, B. M. Fendly, and D. J. Capon.** 1990. Mapping the CD4 binding site for human immunodeficiency virus by alanine-scanning mutagenesis. Proc. Natl. Acad. Sci. USA **87:**7150–7154.
7. **Barlow, P., A. Steinkasserer, D. B. Norman, B. Kieffer, A. P. Wiles, R. B. Sim, and I. D. Campbell.** 1993. Solution structure of a pair of complement modules by nuclear magnetic resonance. J. Mol. Biol. **232:**268–284.
8. **Bartz, R., U. Brinckmann, L. M. Dunster, B. Rima, V. ter Meulen, and J. Schneider-Schaulies.** 1996. Mapping amino acids of the measles virus hemagglutinin responsible for receptor (CD46) down-regulation. Virology **224:** 334–337.
9. **Bowman, M. R., K. D. MacFerrin, S. L. Schreiber, and S. J. Burakoff.** 1990. Identification and structural analysis of residues in the V1 region of CD4 involved in interaction with human immunodeficiency virus envelope glycoprotein gp120 and class II major histocompatibility complex molecules. Proc. Natl. Acad. Sci. USA **87:**9052–9056.
10. **Brodsky, M. H., M. Warton, R. M. Myers, and D. R. Littman.** 1990. Analysis of the site in CD4 that binds to the HIV envelope glycoprotein. J. Immunol. **144:**3078–3086.
11. **Buchholz, C. J., U. Schneider, P. Devaux, D. Gerlier, and R. Cattaneo.** 1996. Cell entry by measles virus: long hybrid receptors uncouple binding from membrane fusion. J. Virol. **70:**3716–3723.
12. **Cho, S.-W., T. J. Oglesby, B.-L. Hsi, E. M. Adams, and J. P. Atkinson.** 1991. Characterization of three monoclonal antibodies to membrane co-factor protein (MCP) of the complement system and quantification of MCP by radioassay. Clin. Exp. Immunol. **83:**257–261.
13. **Clayton, L. K., R. E. Hussey, R. Steinbrich, H. Ramachandran, V. Husain, and E. L. Reinherz.** 1988. Substitution of murine for human CD4 residues identifies amino acids critical for HIV-gp120 binding. Nature **335:**363–366.
14. **Devaux, P., B. Loveland, D. Christiansen, J. Milland, and D. Gerlier.** 1996. Interactions between the ectodomains of haemagglutinin and CD46 as a primary step in measles virus entry. J. Gen. Virol. **77:**1477–1481.
15. **Dörig, R. E., A. Marcil, A. Chopra, and C. D. Richardson.** 1993. The human CD46 is a receptor for measles virus (Edmonston strain). Cell **75:**295–305.
16. **Dörig, R. E., A. Marcil, and C. D. Richardson.** 1994. CD46, a primate-specific receptor for measles virus. Trends Microbiol. **2:**312–318.
17. **Earl, P. L., O. Elroy-Stein, and B. Moss.** 1995. Expression of proteins in mammalian cells using vaccinia viral vectors, p. 16.15–16.19. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
18. **Enders-Ruckle, G.** 1965. Methods of determining immunity, duration, and character of immunity resulting from measles. Arch. Gesamte Virusforsch. **16:**182–207.
19. **Fraser, K. B., and S. J. Martin.** 1978. Measles virus and its biology. Academic Press Inc. (London), Ltd, London, United Kingdom.
20. **Funahashi, S., and T. Kitawaki.** 1963. Studies on measles virus hemagglutination. Biken J. **6:**73–96.
21. **Gerlier, D., B. Loveland, G. Varior-Krishnan, B. Thorley, I. F. C. McKenzie, and C. Rabourdin-Combe.** 1994. Measles virus receptor properties are shared by several CD46 isoforms differing in extracellular regions and cytoplasmic tails. J. Gen. Virol. **75:**2163–2171.
22. **Gerlier, D., G. Varior-Krishnan, and P. Devaux.** 1995. CD46-mediated virus entry: a first key to host-range specificity. Trends Microbiol. **3:**338–345.
23. **Gould, E. A., S. L. Cosby, and P. V. Shirodaria.** 1976. Salt-dependent haemagglutinating measles virus in SSPE. J. Gen. Virol. **33:**139–142.
24. **Graves, M., S. Silver, and P. W. Choppin.** 1978. Measles virus polypeptide synthesis in infected cells. Virology **86:**254–263.
25. **Griffin, D. E., and W. J. Bellini.** 1996. Measles virus, p. 1267–1312. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Virology, 3rd ed. Lippincott-Raven Publishers, New York, N.Y.
26. **Gubler, U., and B. J. Hoffman.** 1983. A simple and very efficient method for generating cDNA libraries. Gene **25:**263–269.
27. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual, p. 55–137. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Hirano, A., S. Yant, K. Iwata, J. Kortesarfaty, T. Seya, S. Nagasawa, and T. C. Wong.** 1996. Human cell-receptor CD46 is down-regulated through recognition of a membrane-proximal region of the cytoplasmic domain in persistent measles virus infection. J. Virol. **70:**6929–6936.
29. **Hsu, E. C., F. Sarangi, and C. D. Richardson.** Unpublished data.
30. **Iwata, K., T. Seya, Y. Yanagi, J. M. Pesando, P. M. Johnson, M. Okabe, S. Ueda, H. Ariga, and S. Nagasawa.** 1995. Diversity of sites for measles virus binding and for inactivation of complement C3b and C4b on membrane cofactor protein CD46. J. Biol. Chem. **270:**15148–15152.
31. **Jameson, B. A., P. E. Rao, L. I. Kong, B. H. Hahn, G. M. Shaw, L. E. Hood, and S. B. H. Kent.** 1988. Location and chemical synthesis of a binding site for HIV-1 on the CD4 protein. Science **240:**1335–1339.
32. **Karp, C. L., M. Wysocka, L. M. Wahl, J. M. Ahearn, P. J. Cuomo, B. Sherry, G. Trinchieri, and D. E. Griffin.** 1996. Mechanism of suppression of cell-mediated immunity by measles virus. Science **273:**228–231.
33. **Kobune, F., H. Sakata, and A. Sugiura.** 1990. Marmoset lymphoblastoid cells as a sensitive host for isolation of measles virus. J. Virol. **64:**700–705.
34. **Kobune, F., H. Takahashi, K. Terao, T. Ohkawa, Y. Ami, Y. Suzaki, N. Nagata, H. Sakata, K. Yamanouchi, and K. Chieko.** 1996. Nonhuman primate models of measles. Lab. Anim. Sci. **46:**315–320.
35. **Krantic, S., C. Gimenez, and C. Rabourdin-Combe.** 1995. Cell-to-cell contact via measles virus haemagglutinin-CD46 interaction triggers CD46 down-regulation. J. Gen. Virol. **76:**2793–2800.
36. **Lalumiére, M., and C. D. Richardson.** 1995. Production of recombinant baculoviruses using rapid screening vectors that contain the gene for β-galactosidase, p. 161–177. *In* C. D. Richardson (ed.), Baculovirus expression protocols. Humana Press, Totowa, N.J.
37. **Lebon, P., A. Protat, and P. Molinie.** 1975. L-Arginine elution of measles virus adsorbed on monkey erythrocytes. Infect. Immun. **11:**1407–1408.
38. **Levy, J. A.** 1994. HIV and the pathogenesis of AIDS, p. 35–36. ASM Press, Washington, D.C.
39. **Lifson, J. D., K. M. Hwang, P. L. Nara, B. Fraser, M. Padgett, N. M. Dunlop, and L. E. Eiden.** 1988. Synthetic CD4 peptide derivatives that inhibit HIV infection and cytopathicity. Science **241:**712–716.
40. **Liszewski, M. K., T. W. Post, and J. P. Atkinson.** 1991. Membrane cofactor protein (MCP of CD46): newest member of the regulators of complement activation gene cluster. Annu. Rev. Immunol. **9:**431–455.
41. **Liszewski, M. K., and J. P. Atkinson.** 1992. Membrane cofactor protein. Curr. Top. Microbiol. Immunol. **178:**45–60.
42. **Maisner, A., J. Schneider-Schaulies, M. K. Liszewski, J. P. Atkinson, and G. Herrler.** 1994. Binding of measles virus to membrane cofactor protein

(CD46): importance of disulfide bonds and *N*-glycans for the receptor function. J. Virol. **68:**6299–6304.
43. **Maisner, A., and G. Herrler.** 1995. Membrane cofactor protein with different types of N-glycans can serve as measles virus receptor. Virology **210:**479–481.
44. **Maisner, A., M. K. Liszewski, J. P. Atkinson, and G. Herrler.** 1996. Oligosaccharide in SCR-2 is required for measles virus binding and infection. J. Virol. **70:**4973–4977.
45. **Malvoisin, E., and T. F. Wild.** 1993. Measles virus glycoproteins: studies on the structure and interaction of the haemagglutinin and fusion proteins. J. Gen. Virol. **74:**2365–2372.
46. **Manchester, M., M. K. Liszewski, J. P. Atkinson, and M. B. A. Oldstone.** 1994. Multiple isoforms of CD46 (membrane cofactor protein) serve as receptors for measles virus. Proc. Natl. Acad. Sci. USA **91:**2161–2165.
47. **Manchester, M., A. Valsamakis, R. Kaufman, M. K. Liszewski, J. Alvarez, J. P. Atkinson, D. M. Lublin, and M. B. A. Oldstone.** 1995. Measles virus and C3 binding sites are distinct on membrane cofactor protein (CD46). Proc. Natl. Acad. Sci. USA **92:**2303–2307.
48. **Mizukami, T., T. R. Fuerst, E. A. Berger, and B. Moss.** 1988. Binding region for human immunodeficiency virus (HIV) and epitopes for HIV-blocking monoclonal antibodies of the CD4 molecule defined by site-directed mutagenesis. Proc. Natl. Acad. Sci. USA **85:**9273–9277.
49. **Naniche, D., T. F. Wild, C. Rabourdin-Combe, and D. Gerlier.** 1993. Measles virus haemagglutinin induces down-regulation of gp57/67, a molecule involved in virus binding. J. Gen. Virol. **74:**1073–1079.
50. **Naniche, D., G. Varior-Krishnan, F. Cervoni, T. F. Wild, B. Rossi, C. Rabourdin-Combe, and D. Gerlier.** 1993. Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. J. Virol. **67:**6025–6032.
51. **Napier, J. R., and P. H. Napier.** 1994. The natural history of the primates, p. 14. MIT Press, Cambridge, Mass.
52. **Nickells, M. W., and J. P. Atkinson.** 1990. Characterization of CR1- and membrane cofactor protein-like proteins of two primates. J. Immunol. **144:** 4262–4268.
53. **Norrby, E.** 1962. Hemagglutination by measles virus. I. The production of hemagglutinin in tissue culture and the influence of different conditions on the hemagglutinating system. Arch. Gesamte Virusforsch. **12:**153–163.
54. **Norrby, E.** 1962. Hemagglutination by measles virus. II. Properties of the hemagglutinin and of the receptors on the erythrocytes. Arch. Gesamte Virusforsh. **12:**164–172.
55. **Norrby, E.** 1963. Hemagglutination by measles virus. III. Identification of two different hemagglutinins. Virology **19:**147–157.
56. **Norrby, E., and M. N. Oxman.** 1990. Measles virus, p. 1013–1044. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology, 2nd ed. Raven Press, New York, N.Y.
57. **Periés, J. R., and C. Chany.** 1961. Activité hémagglutinante et hémolytique du virus morbilleux. C. R. Acad. Sci. **251:**820–821.
58. **Periés, J. R., and C. Chany.** 1961. Mécanisme de l'action hémagglutinante des cultures de virus morbilleux. C. R. Acad. Sci. **252:**2956–2957.
59. **Periés, J. R., and C. Chany.** 1962. Studies on measles viral hemagglutination. Proc. Soc. Exp. Biol. Med. **110:**477–482.
60. **Peterson, A., and B. Seed.** 1988. Genetic analysis of monoclonal antibody and HIV binding sites on the human lymphocyte antigen CD4. Cell **54:**65–72.
61. **Petty, K. J.** 1995. Metal-chelate affinity chromatography, p. 10.11.10–10.11.24. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular virology. John Wiley & Sons, New York, N.Y.
62. **Post, T. W., M. K. Liszewski, E. M. Adams, I. Tedja, E. A. Miller, and J. P. Atkinson.** 1991. Membrane cofactor protein of the complement system: alternative splicing of serine/threonine/proline-rich exons and cytoplasmic tails produce multiple isoforms that correlate with protein phenotype. J. Exp. Med. **174:**93–102.
63. **Purcell, D. F. J., S. M. Russell, N. J. Deacon, M. A. Brown, D. J. Hooker, and I. F. C. McKenzie.** 1991. Alternatively spliced RNAs encode several isoforms of CD46 (MCP), a regulator of complement activation. Immunogenetics **33:**335–344.
64. **Repke, H., D. Gabuzda, G. Palu, R. Emmrich, and J. Sodrosky.** 1992. Effect of CD4 synthetic peptides on HIV type 1 envelope glycoprotein function. J. Immunol. **149:**1809–1816.
65. **Richardson, C. D., A. Scheid, and P. W. Choppin.** 1980. Specific inhibition of paramyxovirus and myxovirus replication by oligopeptides with amino acid sequences similar to those at the N-termini of F1 and HA2 viral polypeptides. Virology **105:**205–222.
66. **Richardson, C. D., A. Berkovich, S. Rozenblatt, and W. J. Bellini.** 1985. Use of antibodies directed against synthetic peptides for identifying cDNA clones, establishing reading frames, and deducing the gene order of measles virus. J. Virol. **54:**186–193.
67. **Richardson, C. D., D. Hull, P. Greer, K. Hasel, A. Berkovich, G. Englund, W. Bellini, B. Rima, and R. Lazzarini.** 1986. The nucleotide sequence of the mRNA encoding the fusion protein of measles virus (Edmonston strain): a comparison of fusion proteins from several different paramyxoviruses. Virology **155:**508–523.
68. **Richardson, C. D., M. Banville, M. Lalumiére, J. Vialard, and E. A. Meighen.** 1992. Bacterial luciferase produced with rapid screening baculovirus vectors is a sensitive reporter for infection of insect cells and larvae. Intervirology **34:**213–227.
69. **Rosanoff, E. I.** 1961. Hemagglutination and hemadsorption of measles virus. Proc. Soc. Exp. Biol. Med. **106:**563–567.
70. **Rota, J. S., K. B. Hummel, P. A. Rota, and W. J. Bellini.** 1992. Genetic variability of the glycoprotein genes of current wild-type measles isolates. Virology **188:**135–142.
71. **Russell, S. M., R. L. Sparrow, I. F. McKenzie, and D. F. Purcell.** 1992. Tissue-specific and allelic expression of the complement regulator CD46 is controlled by alternative splicing. Eur. J. Immunol. **22:**1513–1518.
72. **Sattentau, Q. J., A. G. Dalgleish, R. A. Weiss, and P. C. L. Beverley.** 1986. Epitopes of the CD4 antigen and HIV infection. Science **234:**1120–1123.
73. **Schneider-Schaulies J., L. M. Dunster, F. Kobune, B. Rima, and V. ter Meulen.** 1995. Differential downregulation of CD46 by measles virus strains. J. Virol. **69:**7257–7259.
74. **Schneider-Schaulies, J., J.-J. Schnorr, U. Brinnckmann, L. M. Dunster, K. Baczko, U. G. Liebert, S. Schneider-Schaulies, and V. ter Meulen.** 1995. Receptor usage and differential downregulation of CD46 by measles virus wild-type and vaccine strains. Proc. Natl. Acad. Sci. USA **92:**3943–3947.
75. **Schneider-Schaulies, J., J.-J. Schnorr, J. Schlender, L. M. Dunster, S. Schneider-Schaulies, and V. ter Meulen.** 1996. Receptor (CD46) modulation and complement-mediated lysis of uninfected cells after contact with measles virus-infected cells. J. Virol. **70:**255–263.
76. **Shirodaria, P. V., E. Dermott, and E. A. Gould.** 1976. Some characteristics of salt-dependent haemagglutinating measles viruses. J. Gen. Virol. **33:**107–115.
77. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
78. **ter Meulen, V., and M. A. Billeter.** 1995. Measles virus. Curr. Top. Microbiol. Immunol. **191:**1–192.
79. **Tsui, P., R. W. Sweet, G. Sathe, and M. Rosenberg.** 1992. An efficient phage plaque screen for the random mutational analysis of the interaction of HIV-1 gp120 with human CD4. J. Biol. Chem. **267:**9361–9367.
80. **Vialard, J., M. Lalumiere, T. Vernet, D. Briedis, G. Alkhatib, D. Henning, D. Levin, and C. D. Richardson.** 1990. Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the β-galactosidase gene. J. Virol. **64:**37–50.
81. **Vialard, J. E., and C. D. Richardson.** 1993. The 1,629-nucleotide open reading frame located downstream of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene encodes a nucleocapsid-associated phosphoprotein. J. Virol. **67:**5859–5866.
82. **Wild, T. F., E. Malvoisin, and R. Buckland.** 1991. Measles virus: both the haemagglutinin and fusion glycoproteins are required for fusion. J. Gen. Virol. **72:**439–442.
83. **Zhang, X., M. Gaubin, L. Briant, V. Srikantan, R. Murali, U. Saragovi, D. Weiner, C. Devaux, M. Autiero, D. Piatier-Tonneau, and M. I. Greene.** 1997. Synthetic CD4 exocyclics inhibit binding of human immunodeficiency virus type 1 envelope to CD4 and virus replication in T lymphocytes. Nat. Biotech. **15:**150–154.