

Cell Entry by Measles Virus: Long Hybrid Receptors Uncouple Binding from Membrane Fusion

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The pH-independent fusion of membranes induced by measles virus (MV) requires, in addition to the fusion-competent protein F, hemagglutinin (H), and on the target membrane, the virus receptor CD46. We constructed hybrid receptors composed of different numbers and combinations of the four CD46 short consensus repeat (SCR) domains, followed by immunoglobulin-like domains of another cell surface protein, CD4. Hybrid proteins containing SCRs I and II bound MV particles and conferred fusion competence to rodent cells. SCRs III and/or IV strengthened MV binding. Increasing the distance between the MV binding site and the transmembrane domain enhanced virus binding but reduced fusion efficiency. A hybrid protein predicted to be about 120 Å (12 nm) longer than the standard receptor lost fusion support function and was dominant negative over a functional receptor. These data indicate that receptor protein length influences virus binding and determines fusion efficiency.

Membrane fusion is essential for many cellular functions including intracellular membrane traffic, fertilization, or polykaryon formation in muscular tissue. The best characterized fusion mechanisms are those of certain enveloped viruses whose fusion proteins are easy to purify and manipulate. Viral envelopes fuse with cellular membranes in a pH-dependent or -independent way. Both fusion processes can be subdivided in two steps: (i) binding of the virus particle to a cellular receptor and (ii) fusion of the two membranes.

The influenza virus HA protein is the fusion factor studied in greatest detail (41, 44). This protein combines both receptor binding and membrane fusion functions. Membrane fusion is triggered by the low pH in the endocytic organelle which induces a conformational change resulting in the insertion of the 24-amino-acid-long hydrophobic fusion peptide into the endosomal membrane (7, 8). Upon membrane fusion, the viral genome is released into the cytoplasm.

The mechanisms of pH-independent fusion, usually fusion at the cell surface, are less well defined. Fusion at the plasma-membrane is characteristic of several viruses including human immunodeficiency virus (HIV), other retroviruses, and all paramyxoviruses (22, 27, 28). This type of fusion has often been studied at the cellular level: cells expressing viral proteins fuse with cells expressing viral receptors, thereby forming multinucleated giant cells. On the basis of this syncytium formation assay, the components of the fusion complex were defined.

In measles virus (MV), as in other paramyxoviruses, both the fusion (F) protein and hemagglutinin (H) are required for fusion (9, 46). These proteins are organized on the viral surface in a regular array of tightly packed spikes, H tetramers, and F trimers (29, 32, 37). The H protein on one hand recognizes the receptor on the target membrane and on the other supports the F protein in the fusion process, which is likely to involve the

insertion of an hydrophobic fusion peptide in the cell membrane (reviewed in reference 22).

Most paramyxoviruses bind cells via sialic acid residues situated on cell surface glycoconjugates. MV is an exception; it uses a single protein, the regulator of complement activation CD46, as its main receptor (12, 30; reviewed in reference 15). CD46 is a type I transmembrane protein which is expressed in four major isoforms (23). These isoforms arise by alternative splicing and differ in the presence or absence of a short domain (STP B) and in having one of two alternative cytoplasmic tails (named 1 and 2). All isoforms contain four short consensus repeat (SCR) domains, modules of about 60 amino acids characteristic for many proteins involved in the regulation of complement activation (23).

The tertiary structure of the CD46 SCR domains has not been determined yet, but nuclear magnetic resonance analysis revealed that SCR modules of factor H have a compact hydrophobic core wrapped in β -strand and sheet. The length of one domain is slightly less than 30 Å (3 nm), and it is expected that all other SCR modules have a very similar secondary structure and length (2, 3). Nuclear magnetic resonance and electron microscopy studies indicate that neighboring SCRs are tilted; thus, the distance between the top and bottom of four domains is estimated to be about 100 Å (10 nm) (4, 11).

Several laboratories have begun to study the nature of the protein-protein interactions underlying MV attachment and MV-induced fusion. In particular, it was recently found that the two membrane-distal SCR domains of CD46 joined to the two membrane-proximal SCRs of CD55 confer MV replication competence to hamster cells (21, 26). Subtle effects of the STP (serine-threonine-proline-rich) domains on MV binding and fusion were also monitored (5, 19).

In the present report on the mechanism of MV entry, we confirmed that SCRs I and II are sufficient for primary virus binding, and we uncovered a secondary contribution of SCR III and/or IV. We also observed that fusion efficiency depends on receptor protein length. Receptor length was changed by transferring SCRs I and II to backbones consisting of immunoglobulin-like (Ig-like) domains of the HIV receptor CD4

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(length of four Ig-like domains, 125 Å [12.5 nm]) (reviewed in reference 17).

MATERIALS AND METHODS

Antibodies, viruses, and cells. In flow cytometry, monoclonal antibody MCI 20.6 (CD46 [30]), monoclonal antibody c155 (H protein [16]), and the OKT4 antibody (CD4 [Becton-Dickinson]) were used. For Western blots (immunoblots), a CD46-specific rabbit serum (gift of K. Liszewski and J. Atkinson), a MV N-protein-specific rabbit serum (serum 179; gift of C. Oervell), and a MV H-specific rabbit serum were used. The latter was raised against a peptide derived from the amino terminus of the H protein, NH₂-SPQRDRIN-AFYKDN(C)-COOH (a cysteine was attached to the carboxyl terminus of the peptide in order to couple to keyhole limpet hemocyanin for rabbit immunization).

The MV Hallé and Edmonston strains were grown in Vero cells (30, 36). Recombinant vaccinia viruses were grown on BHK 21 cells. The vaccinia virus coding for the MV F and H proteins (VV-F/H) was kindly provided by R. Drillien and described previously (45). The vaccinia virus vTF7-3 coding for bacteriophage T7 RNA polymerase was kindly provided by B. Moss (14).

Mouse Ltk⁻ cells and CHO cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM-10% FCS). CHO cells expressing CD46 (BC1 isoform) were kindly provided by K. Liszewski and J. Atkinson and have been described elsewhere (25).

Generation and MV infection of stable cell lines. For the generation of stably expressing CHO cells, plasmids were introduced by the calcium phosphate precipitation method. Briefly, cells were grown in 8.5-cm-diameter petri dishes. Fifteen micrograms of plasmid DNA was dissolved in 250 µl of 0.25 M CaCl₂ and mixed with 250 µl of HeBS (1.5 mM Na₂HPO₄, 50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 280 mM NaCl) (pH 7.05). The precipitate was added to the cells and removed 24 h later. Selection of transfected cells with G418 was started after another 24 h. Transfectants expressing similar amounts of the chimeric proteins were chosen from clones which arose from single cells sorted with MCI 20.6. Cell lines were cultivated in DMEM-10% FCS containing 1 mg of G418 per ml.

Cells were infected with the MV Edmonston strain at a multiplicity of infection of one, scraped into the medium about 40 h after infection, and lysed by two freeze-thaw cycles. Cell debris were removed by centrifugation, and MV titers were determined by plaque assay on Vero cells: virus was absorbed, cells were overlaid with 1% SeaPlaque agarose in DMEM-2% FCS, incubated for 5 days, fixed with 10% trichloroacetic acid, and stained with crystal violet, and the plaques were counted.

Generation of plasmids coding for hybrid CD46-CD4 proteins. Plasmid pRcCD46 (5) bears a CD46-BC1 cDNA under control of both the T7 and cytomegalovirus promoters, making transient and stable expression possible. This plasmid was used as backbone for insertion of DNA segments coding for CD46 and CD4 (24) domains with modified boundaries. The CD46 and CD4 DNA segments were generated by PCR (38). Primers were designed to allow the combination of PCR amplicons by ligation and to code for a 5-amino-acid-long glycine-rich peptide (glycine hinge) between the CD46 and CD4 parts. The plasmid coding for the chimeric protein II+I/4 was generated by overlap extension PCR (18). All constructs were sequenced to verify that no undesired mutations had been introduced.

The boundaries of the SCR and Ig-like domains of the hybrid proteins were the following (numbering as in references 23 and 24; EMBL/GenBank accession no. M35160): SCR I, C-35 to E-97, when located downstream of the leader peptide, and A-34 to E-97, when placed downstream of SCR II; SCR II, T-98 to V-161, when present as the second domain, but C-99 to V-161, when placed downstream of the leader peptide. The CD46 part of hybrids containing all four SCRs extended to V-286. Between the CD46 and CD4 segments, all chimeric proteins contained a glycine hinge, GGGLG for proteins containing only the fourth Ig-like domain of CD4 and GGGGA for all other chimeric proteins. The CD4 segments in the chimeric proteins started with S-368 for hybrids without any Ig-like domain, with R-295 for hybrids containing the fourth Ig-like domain, with A-180 for hybrids containing the third and fourth Ig-like domains, and with K-3 for hybrids containing all four Ig-like domains. All chimeric proteins contained the CD46 leader peptide (M-1 to A-34).

Transfection procedures; protein analysis by Western blotting. For transient expression in mouse Ltk⁻ cells, the vaccinia virus T7 expression system was used (14). About 10⁶ cells were infected with 10⁷ PFU of vTF7-3 and then transfected with 3 µg of plasmid DNA using 8 µl of lipofectin (GIBCO). Cells were harvested about 18 h after transfection for Western blot or flow cytometric analysis. For Western blots, cytoplasmic cell extracts were prepared as previously described (6), separated on sodium dodecyl sulfate-10% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were incubated with the polyclonal anti-CD46 serum at a dilution of 1:4,000. Bound antibodies were detected with a peroxidase-conjugated second antibody (Dakopats) by using the ECL system (Amersham).

MV binding assay and flow cytometry. For binding assays (30), MV particles of the Hallé strain were purified over a sucrose gradient. Cells were detached from 6-well dishes 18 h after transfection by phosphate-buffered saline (PBS)-EDTA treatment. About 2 × 10⁵ cells were incubated with an excess of

purified MV particles at 37°C for 1 h in DMEM-6% FCS supplemented with 0.05% NaN₃. Cells were washed twice and then incubated for 30 min with the H-specific monoclonal antibody c155, which was detected with a phycoerythrin-conjugated anti-mouse antibody (Immunotech). CD46-independent binding was measured after transfecting the cells with a pRc/CMV vector containing the CD46 open reading frame in reverse orientation. Cell surface expression of the chimeric proteins was determined by using the SCR I-specific monoclonal antibody MCI 20.6 (30, 47) or polyclonal antiserum for the two hybrids not encoding SCR I. Binding efficiencies were obtained by dividing the binding values (mean fluorescence of specific binding minus mean fluorescence of background binding) by the levels of surface expression. Measurements were in triplicate.

Control experiments included the use of binding assay supernatants to reprobe binding. Binding levels were similar to those found in the first assay, confirming that the system was saturated with virus. Moreover, other monoclonal antibodies confirmed the results obtained with MCI 20.6, indicating that potential conformation effects did not influence our measurements. As a further control, the transfected cells were incubated with purified MV particles heat inactivated for 30 min at 60°C. Heat inactivation inhibits MV infectivity but not binding (34). Heat-inactivated MV loses reactivity with two monoclonal anti-F antibodies but not with the anti-H c155 antibody (10a). Binding results were slightly higher with heat-inactivated virus than with standard virus, but the same ratios were obtained. Finally, control binding assays performed at 4°C were less efficient than those performed at 37°C, but again the same ratios were obtained. Thus, membrane fusion or endocytosis, if it occurs at all, was not relevant for our MV binding measurements.

Cell-cell fusion assays. Four hours after transfection, about 8 × 10⁵ cells were detached with PBS-EDTA and cocultivated with an equivalent number of Ltk⁻ cells, infected with 10 PFU of VV-F/H per cell. The formation of syncytia was monitored 15 h after the start of cocultivation. The fusion efficiency was determined by counting nuclei in syncytia and total nuclei. Levels of protein expression were controlled by Western blot analysis from extracts of the cocultivated cells prepared after determination of the fusion levels.

RESULTS

CD46 domains involved in MV binding and MV-induced fusion. Since the binding domains of viral receptors are often situated near the membrane-distal protein end (for a review, see reference 48), we included SCR I in the first three CD46-CD4 hybrid proteins constructed (Fig. 1B, bottom). Hybrid I/4 (protein 2) (SCR domains in proteins indicated by roman numerals and CD4 domains indicated by arabic numerals) contained SCR I joined to the fourth Ig-like module and to the CD4 transmembrane and intracellular domains, hybrid I-II/4 (protein 3) contained SCRs I and II, and hybrid I-IV/4 (protein 4) contained all four SCRs joined to the same CD4 segment, respectively. Four glycine residues were inserted at the boundary between the SCRs and the Ig-like segments to allow for structural flexibility.

For the analysis of MV receptor properties, these three proteins were transiently expressed in mouse Ltk⁻ cells. As a negative control, the CD4 protein was also produced (Fig. 1A). Western blot analysis of cell extracts revealed that all proteins were expressed at similar levels and had the expected molecular weight (data not shown). Surface expression and MV binding were quantified by flow cytometry as described previously (30).

Cells expressing the hybrid protein with SCRs I and II and cells expressing the hybrid protein with all four SCRs bound MV (Fig. 1A, I-II/4 and I-IV/4 proteins). Cells expressing the hybrid protein with only SCR I showed MV binding within the background range, as did control cells expressing CD4 (Fig. 1A, I/4 and CD4) (additional control experiments as in Materials and Methods). We note that two cell populations with different expression levels were reproducibly detected with all the CD46-CD4 hybrids and with the CD46-BC1 standard.

In Fig. 1B, the binding efficiencies (mean binding values divided by mean surface expression) of the three hybrids are represented by black columns and compared with the binding efficiency of the CD46-BC1 isoform, which was set at 100%. The hybrid with all four SCRs bound MV at a level more than double that of CD46-BC1, whereas the hybrid with SCRs I and

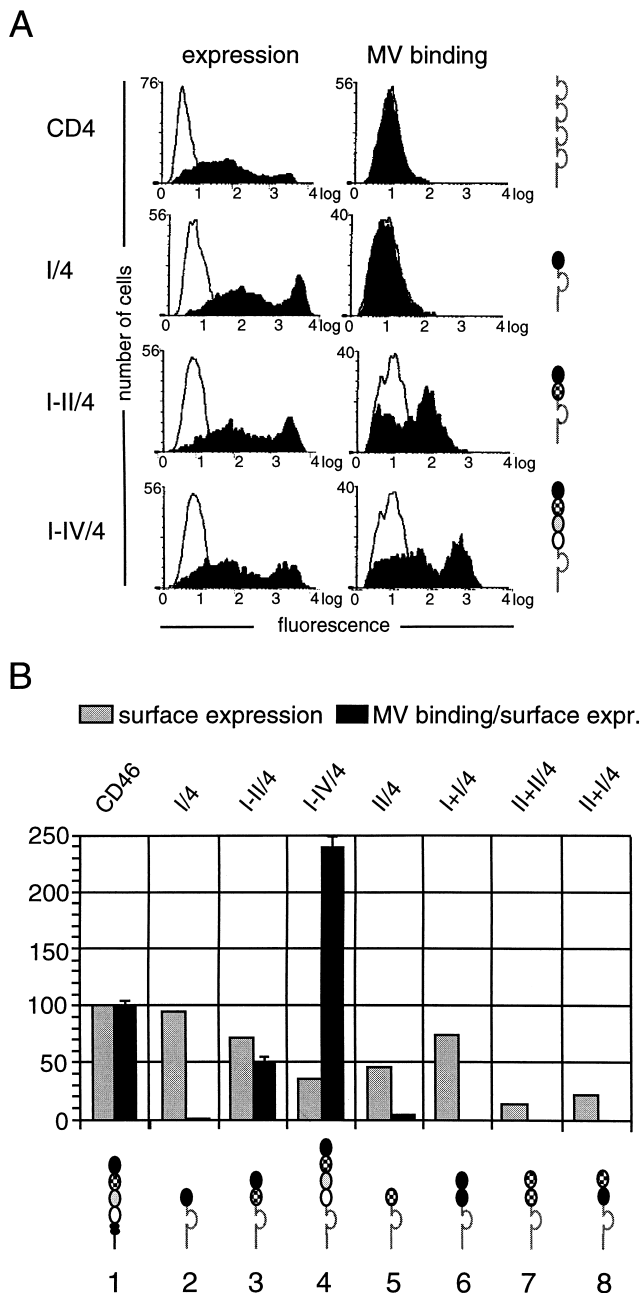


FIG. 1. Analysis of MV binding to seven CD46-CD4 protein hybrids. (A) Expression and binding profiles of CD4 and of three CD46-CD4 hybrids expressed in Ltk⁻ cells by using the VV-T7 system. All measurements were performed from pools of transfected cells split into aliquots. Surface expression was determined with the monoclonal anti-CD46 antibody MCI 20.6 (proteins I/4, I-II/4, and I-IV/4) or a CD4-specific monoclonal antibody (left panels, black profiles). As a control, cells were incubated only with the second antibody (white profiles). For MV binding, cells were incubated with purified MV in the presence of 0.05% NaN₃, and bound MV particles were detected by using the anti-H antibody cI55 (black profiles). The MV binding profiles of the transfected cells were superimposed on the binding profile of control-transfected, VV-T7-infected Ltk⁻ cells (white profiles). Fluorescence intensity on the horizontal axis has a logarithmic scale. To the right of each set of graphs (and in all subsequent figures), the proteins are depicted by using the following conventions. The CD46 part is shown in black, and the CD4 part is shown in grey. The SCR I filling is black, that of SCR II is crosshatched, that of SCR III is light grey, and that of SCR IV is white. (B) Surface expression and MV binding efficiencies of seven CD46-CD4 chimeric proteins compared with the standard, CD46-BC1. Based on the flow cytometry data, surface expression (grey columns) and MV binding efficiencies (black columns) were calculated for each protein hybrid and are shown in percentages of the levels scored by the CD46-BC1 isoform. MV binding

II bound MV at a level half that of the standard. These data indicate that two membrane-distal SCRs are sufficient for MV binding.

Next we asked if SCR II alone could bind virus. A hybrid with SCR II joined to the fourth CD4 Ig-like domain was constructed (II/4 or protein 5 [Fig. 1B]). To take into account possible distance effects, we constructed a hybrid with a duplicated SCR II (II+II/4 [protein 7]) and in an analogous manner, a duplicated SCR I hybrid (I+I/4 [protein 6]). We also constructed a hybrid with SCRs I and II in the inverted order (II+I/4 [protein 8]). These four hybrid proteins were expressed at the cell surface (grey columns in Fig. 1B) but did not bind virus (indicated by the absence of black columns [in this experiment proteins 7 and 8 were expressed at levels only 30 to 40% that of the reference protein, but in other experiments with higher surface expression, lack of binding was confirmed]). These results indicate that SCR II alone cannot bind virus and suggest that SCRs I and II do not contain an exchangeable, duplicated MV binding site.

Next we tested the seven hybrid proteins for the ability to mediate cell-cell fusion in a cocultivation assay. Mouse Ltk⁻ cells transiently expressing hybrid receptor proteins were mixed with an equal number of Ltk⁻ cells expressing the MV F and H proteins. Figure 2 shows that both hybrid proteins efficiently binding MV also supported MV-induced fusion. In cells expressing the most efficient mediator of virus binding, hybrid I-IV/4, 20% of the nuclei were in syncytia, with 10 to 30 nuclei per syncytium. Surprisingly, in cells expressing the less strongly binding I-II/4 protein, 70% of the nuclei were in syncytia with up to 100 nuclei per syncytium. In control cocultures of cells expressing the CD46-BC1 isoform, about 10% of the nuclei were in small syncytia, with 5 to 10 nuclei per syncytium. Cells expressing the five hybrid proteins not binding MV did not induce syncytium formation (I/4 in Fig. 2 and data not shown).

The higher efficiency in supporting fusion of the CD46-CD4 hybrid proteins compared with the CD46 standard is due at least in part to the obliteration of the two short, heavily O-glycosylated CD46 BC domains, which negatively influence cell fusion (5, 20). The difference in fusion efficiency between the I-II/4 and I-IV/4 proteins was unexpected.

Increasing the receptor protein length has the opposite effect on binding and fusion. The above observations could be accounted for by an opposite dependency of virus binding and MV-induced fusion on receptor length. This hypothesis was tested with six new hybrid proteins (Fig. 3). To reduce receptor length, SCRs I and II were directly linked to the CD4 transmembrane domain (I-II and I-IV hybrids [proteins 2 and 6]) by eliminating one Ig-like domain (30 Å long) from the available hybrid proteins I-II/4 and I-IV/4 (proteins 3 and 7). To increase receptor length, one (I-II/3-4 and I-IV/3-4 [proteins 4 and 8]) or three (I-II/1-4 and I-IV/1-4 [proteins 5 and 9]) Ig-like domains were inserted. All the hybrid proteins were transported to the cell surface (Fig. 3, grey columns).

As expected, all the hybrid receptors bound MV but with different efficiencies. The shortest molecule, that is, the I-II hybrid protein, exhibited the lowest binding efficiency (protein 2, about 10% of that of the control CD46-BC1 molecule [protein 1]). On the other hand, the long proteins with SCR III and

efficiencies were calculated by dividing MV binding by cell surface expression. MV binding was measured in triplicate, and the mean deviation of the measurements is indicated at the top of the column. The two dots below the CD46-BC1 (protein 1) represent the B and C STP domains.

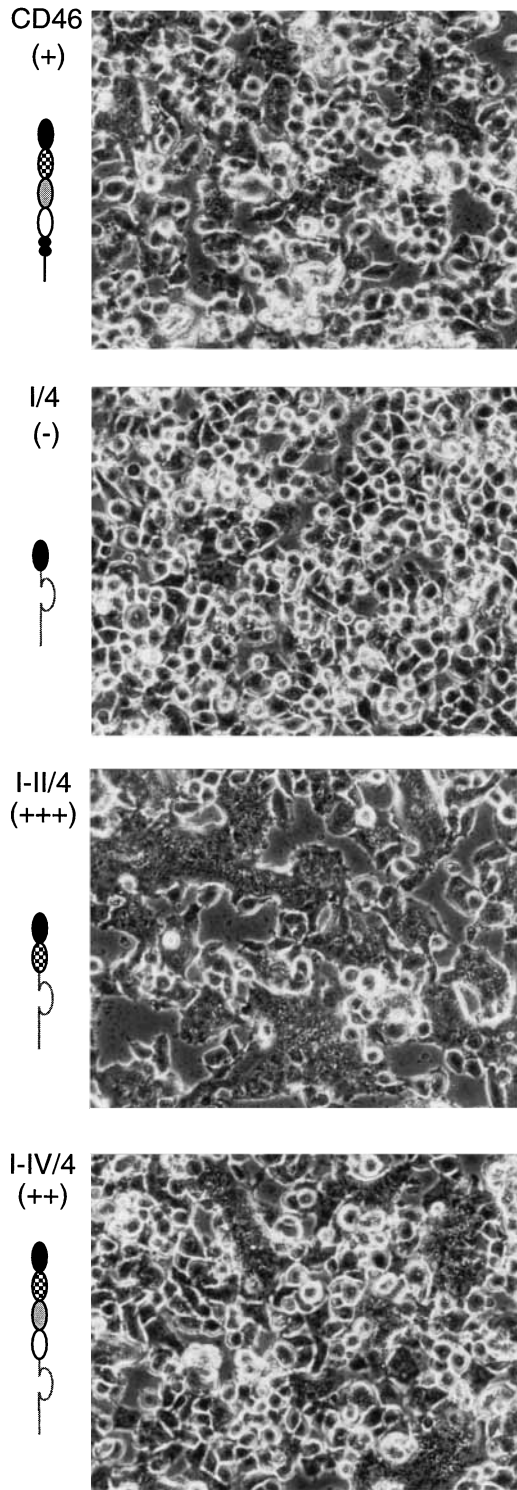


FIG. 2. Cell-cell fusion after cocultivation of cells expressing CD46-CD4 hybrid proteins with cells expressing the MV F and H proteins. The CD46-BC1 isoform and the hybrid proteins I/4, I-II/4, and I-IV/4 were expressed in mouse Ltk⁻ cells by using the VV-T7 system. Four hours after transfection, cells were detached and cocultivated with an equal number of MV F/H-expressing Ltk⁻ cells. Syncytium formation was monitored 15 h later. The fusion efficiency was determined by counting nuclei in syncytia and total nuclei. Symbols: +, 5 to 15% of the nuclei in syncytia, with 5 to 10 nuclei per syncytium; ++, 20 to 30% of the nuclei in syncytia, with 10 to 30 nuclei per syncytium; +++, >40% of the nuclei in syncytia, with up to 100 nuclei per syncytium.

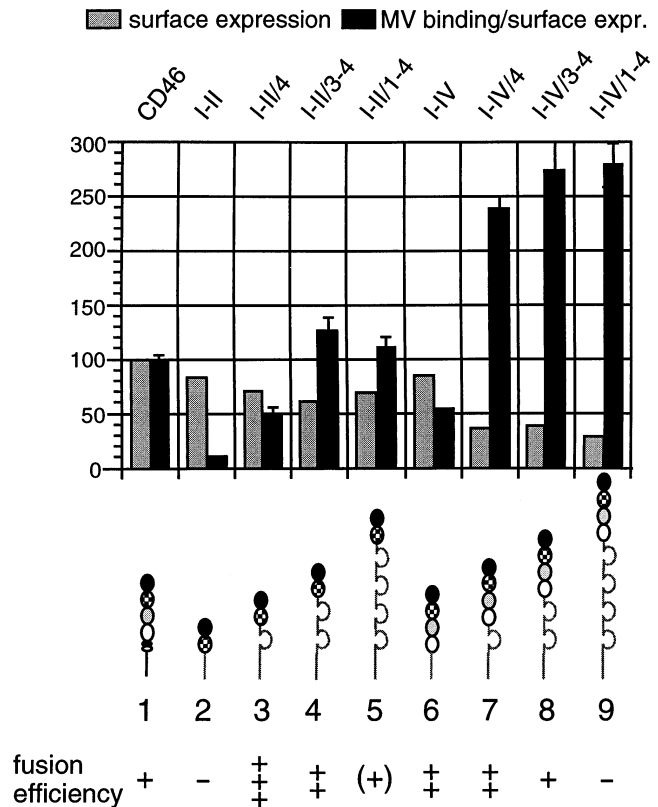


FIG. 3. Surface expression and MV binding and fusion support efficiencies of CD46-CD4 hybrid proteins of different lengths. Proteins were expressed in Ltk⁻ cells by using the VV-T7 system. MV binding was measured with the hemagglutinin-specific antibody c155. Binding efficiencies were calculated by dividing the binding values by surface expression and are shown in relation to that of the standard (CD46-BC1). Surface expression levels varied up to 15% in different experiments. MV binding efficiency was measured in triplicate, and the mean deviation is indicated at the top of the column. The fusion efficiencies of the different hybrid proteins are indicated at the bottom by the convention as explained in the legend to Fig. 2.

IV were very efficient in binding, reaching levels two to three times higher than those of the CD46 standard (proteins 7, 8, and 9).

Comparison of the binding efficiencies of hybrids with all SCRs (proteins 6 to 8) with those of corresponding hybrids of similar size with only SCRs I and II (proteins 3 to 5), indicates that SCRs III and IV enhanced binding more than the Ig-like domains, the exception being the protein couple 3 and 6.

The hybrids were then tested for the ability to support membrane fusion in a cocultivation assay. Apart from the weakly binding I-II hybrid (protein 2) (Fig. 3), all other hybrids bound MV strongly and could therefore be expected to efficiently support fusion. This was the case, but with two exceptions: when SCRs I and II were separated from the membrane by four CD4 Ig-like domains (125 Å [12.5 nm]), syncytium formation was just above the background level (protein 5). Insertion of two more SCRs (50 Å [5.0 nm]) resulted in the complete loss of fusion competence (protein 9). When the fusion efficiencies of molecules with one, two, or four Ig-like domains are compared (e.g., proteins 3, 4, and 5 or proteins 7, 8, and 9), an inverse correlation with the protein size becomes evident. Thus, increasing protein length indeed enhances binding but reduces fusion, and only proteins of intermediate length maintain efficient binding and fusion functions.

A long hybrid receptor is dominant negative in fusion. We then asked if long receptor molecules could compete with an excess of functional receptor molecules in a fusion assay. Ltk^- cells were cotransfected with a plasmid coding for a fusion-support-deficient receptor (I-IV/1-4) and another coding for a functional receptor (I-II/4) in a 1-to-5 molar ratio. As controls, cells were cotransfected with a plasmid coding for a nonbinding, fusion-support-deficient protein (I/4) and with the I-II/4 plasmid in a 2-to-1 molar ratio.

Figure 4 shows that cells expressing the fully functional protein and the fusion-support-deficient protein did not fuse (Fig. 4A), whereas cells producing the fully functional protein and the nonbinding and fusion-deficient proteins formed syncytia (Fig. 4B). The expression of the hybrid proteins was analyzed by Western blotting. As expected, about five times less I-IV/1-4 protein than I-II/4 protein was produced (Fig. 4C, lane A). Even at this unfavorable molar ratio, the long I-IV/1-4 receptor exerted a dominant negative effect.

MV propagation in CHO cells stably expressing hybrid receptors. To test the capacity of hybrid receptors to support stages of viral propagation after entry and to assay virus-cell fusion directly, we established stable cell lines. CHO cells were chosen because after CD46 expression they become permissive for MV infection, unlike mouse Ltk^- cells (12, 30). Two cell lines expressing proteins strongly or completely impaired in fusion support function (Fus^- receptors I-IV/1-4 and I-II/1-4) and three cell lines expressing fully functional proteins (Fus^+ receptors I-II/4, I-II/3-4, and I-IV/3-4) were generated. As expected, all these cell lines bound MV, as shown in Fig. 5A for a Fus^+ receptor (I-II/4) and a Fus^- receptor (I-IV/1-4). Consistent with the results obtained with transient expression in Ltk^- cells, the Fus^- receptor bound MV more efficiently than the Fus^+ receptor.

The cell-cell and virus-cell fusion competence of the cell lines was then tested. CHO cells expressing the Fus^+ receptors fused efficiently with Ltk^- cells, while cells expressing the Fus^- receptors did not fuse (data not shown), corroborating the results obtained by the transient expression analysis.

We then infected the three cell lines expressing a Fus^+ receptor and the two cell lines expressing a Fus^- receptor with MV and monitored the course of infection by microscopy. As documented in Fig. 5B for the I-II/4 Fus^+ receptor cell line, 40 h after infection, cells formed large syncytia, which detached within a few hours. In contrast, the level of fusion of MV-infected Fus^- receptor cell lines (e.g., I-IV/1-4 [Fig. 5C]) did not exceed the low level of syncytium formation of uninfected cells, and no cytopathic effect was observed. Fus^- cells formed syncytia upon infection with the close MV relative canine distemper virus, which does not use CD46 as a receptor (33). Thus, MV entry in Fus^- cell lines is significantly less efficient than in standard CHO cells, suggesting that long hybrid receptors interfere with an available alternative entry pathway, perhaps by sequestering MV particles.

To verify the extent of viral propagation in Fus^+ and Fus^- receptor cells, extracts were prepared 40 h after infection and assayed for the viral H and nucleocapsid (N) proteins. The three Fus^+ receptor cell lines produced both proteins, whereas the two Fus^- receptor cell lines did not (Fig. 6) (after long exposure, faint N and H signals became visible in CHO cells but not in the Fus^- receptor cell lines). These observations imply that MV can efficiently enter only Fus^+ receptor cell lines and thus that virus-cell fusion has the same receptor requirements as cell-cell fusion.

We then analyzed virus production in two Fus^+ receptor cell lines in which the early MV replication stages are about as efficient as in CHO-CD46 cells, as monitored by the N- and

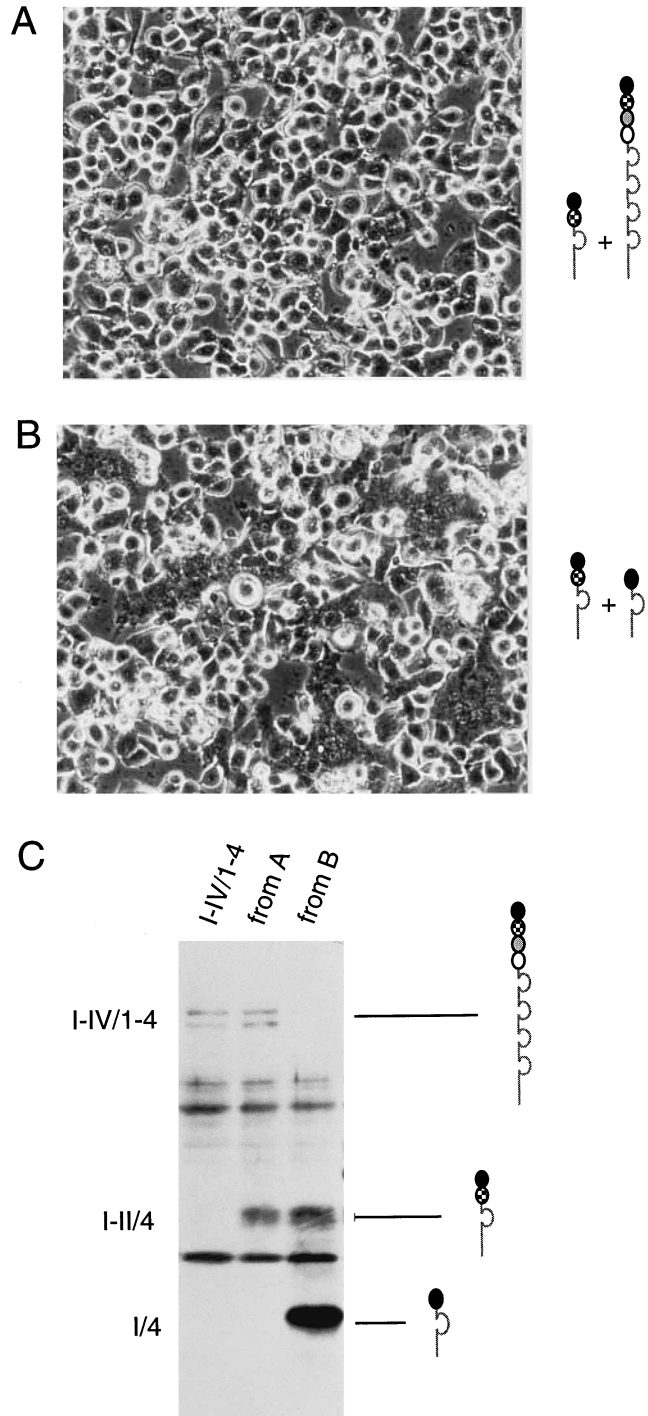


FIG. 4. The hybrid protein I-IV/1-4 is dominant negative in fusion support over the hybrid protein I-II/4. (A) Ltk^- cells transfected with two plasmids: one (0.5 μ g of DNA) coding for the I-IV/1-4 protein and the other (2.5 μ g of DNA) coding for the I-II/4 protein. Cells were detached 4 h after transfection, cocultivated with an equivalent number of F- and H-expressing Ltk^- cells, and photographed after another 15 h. (B) Control Ltk^- cells transfected with two plasmids: one (2 μ g of DNA) coding for the I/4 protein and the other (1 μ g of DNA) coding for the I-II/4 protein, as described above. (C) Amounts of the hybrid proteins in the cotransfected cells. Cytoplasmic extracts from the cocultivated cells shown in panels A and B were separated on a sodium dodecyl sulfate-10% polyacrylamide gel and analyzed by Western blotting. Extracts from cells transfected only with the plasmid encoding the I-IV/1-4 protein were used as a control (lane I-IV/1-4). The positions of the three hybrid proteins are indicated.

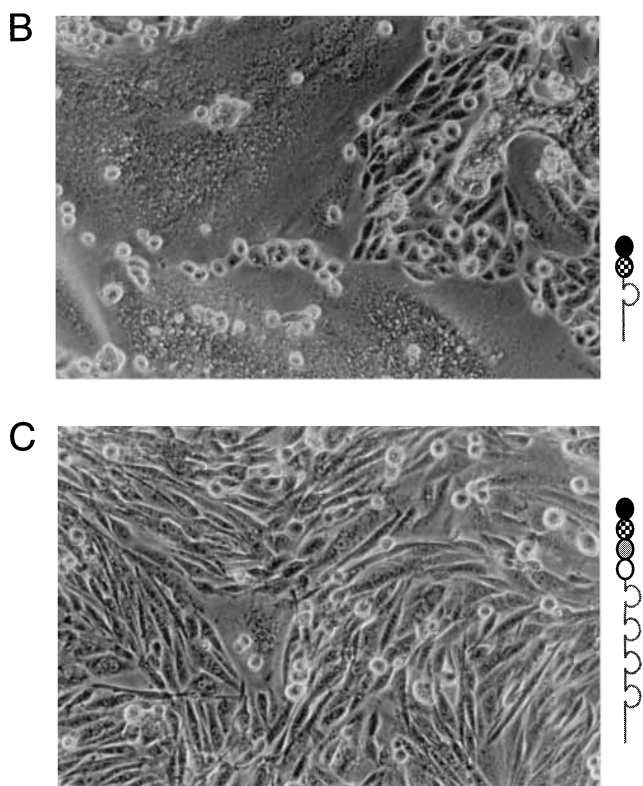
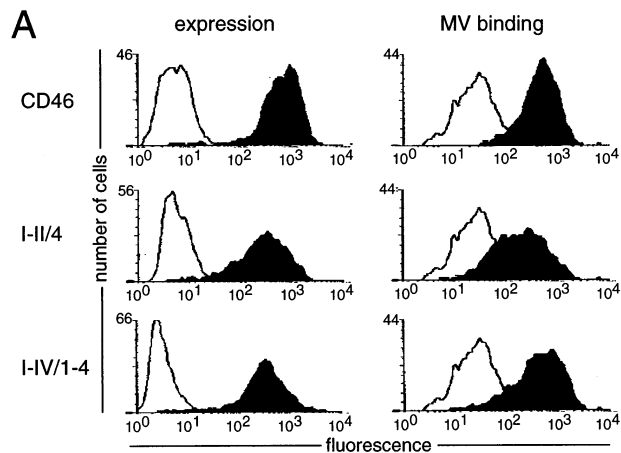


FIG. 5. MV binding (A) and syncytium formation (B and C) of CHO cells stably expressing the hybrid protein I-II/4 or I-IV/1-4. Surface expression was monitored by using the monoclonal anti-CD46 antibody MCI 20.6 (black profiles). Expression profiles were superimposed on those obtained for the second antibody alone (white profiles). Binding of fusion-inactive MV particles was detected with the anti-H monoclonal antibody c155 (black profiles), and the binding profiles were superimposed on that of untransfected CHO cells (white profiles). Syncytium formation was documented 40 h after inoculation with about one infectious virus particle per cell. The small syncytium in panel C is characteristic of MV-independent cell fusion, as similar syncytia were observed in uninfected CHO cells.

H-protein expression levels (Fig. 6 and data not shown). In I-II/4 and I-II/3-4 cells, MV titers were 10⁴ and 2 × 10⁴ (Fig. 6). These titers were only two and four times higher, respectively, than in wild-type CHO cells but 25 to 50 times lower than in CHO-CD46 cells. Thus, cells expressing hybrid MV receptors which efficiently sustain the production of viral proteins are

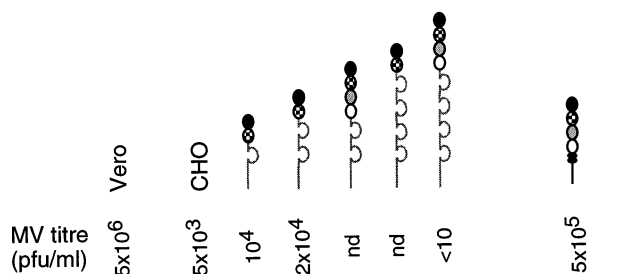
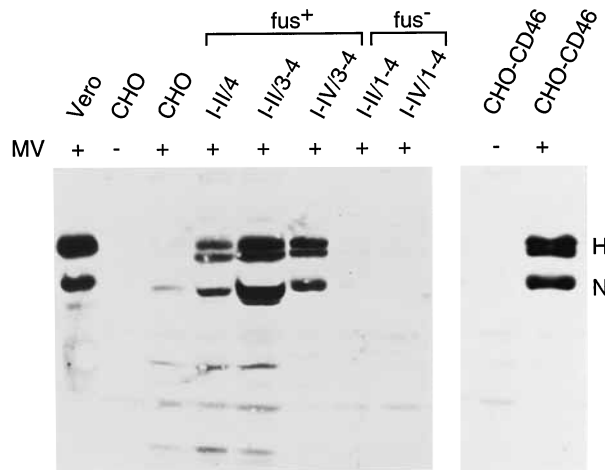


FIG. 6. Propagation of MV in CHO cell lines expressing different hybrid CD46-CD4 receptors. Vero, CHO, and CHO-derived cell lines were infected with one infectious particle per cell (+) or not infected (-). CHO and CHO-derived cells were harvested 40 h after infection, and Vero cells were harvested 24 h after infection. For Western blot analysis, equal amounts of protein were loaded for all CHO and CHO-derived cells; four times less material from Vero cells was used. The data from CHO-CD46 cells are from another gel and not directly comparable. The positions of the MV N and H proteins are indicated. MV titers released from the infected cells are shown at the bottom (nd, not determined).

poor virus producers. This suggests that the hybrid proteins are not able to support or interfere with late viral replication steps such as assembly or budding.

DISCUSSION

We show that an increase in the length of receptor molecules has different effects on MV binding and virus-induced membrane fusion. Standard-size and long receptors bind MV efficiently, with long molecules binding above standard levels. However, only proteins of approximately standard size support fusion efficiently. This optimal length effect, the observation that a long receptor molecule is dominant negative in fusion, and the mode of virus binding have a bearing on the mechanism of MV entry.

MV binding to CD46. We confirmed the recent observation that CD46 SCRs I and II are essential for MV binding (21, 26) and extended it in three ways. First, we observed that CD46-CD4 hybrids containing a duplication of SCR I or SCR II or an inversion of these two domains cannot bind MV. These results could be due to a structural alteration of the SCRs in these hybrid proteins or, more interestingly, could suggest that the MV binding site is located at their interface. The last hypothesis is currently under experimental analysis. On the basis of the known structure of two SCR domains of factor H (4), a three-dimensional model of the CD46 MV-binding domains

has been produced (29a), and residues predicted to map in the interface region are being mutated (21a).

Second, we observed that inserting an increasing number of spacer (Ig-like) domains in receptor molecules resulted in enhancement of virus binding. We cannot yet draw firm conclusions about the structure and physical length of the hybrid proteins, but since at least two of the three CD4 Ig domain interfaces are relatively rigid (17), we consider it likely that insertion of the Ig-like domains results in moving the MV binding site away from the membrane. It is thus conceivable that enhancement of binding results from a reduction of steric hindrance. Steric hindrance was responsible for the decrease of C4b binding to CD46 when an SCR was deleted (1). Long CD46-CD55 hybrids with eight SCRs have been constructed (19), and it will be interesting to study their MV receptor function. In this context we note that SCR domain interfaces have swivelling freedom (4, 11), which could influence binding and/or fusion.

Third, we observed that the addition of SCRs III and IV to the MV binding site enhanced MV binding more than the addition of Ig-like domains of similar size, suggesting either that one of these domains binds the virus secondarily or that SCRs III and IV influence the disposition of SCRs I and II. This result is in line with the observation of Iwata et al. (21) that deletion of SCR IV reduced MV binding. Interestingly, SCRs III and IV did not enhance the binding efficiency of a recombinant soluble dimeric form of purified MV H protein (10a). This suggests that SCRs III and IV stabilize binding only after the formation of a multimeric complex (see below).

There is an overlap between the CD46 domains involved in primary or secondary interactions with MV and those interacting with complement proteins (1, 21). As a consequence, MV and the physiological CD46 ligands are likely to compete for binding, whereby MV infection would induce complement activation (40). MV infection is already known to affect CD46 function through downregulation of surface expression (31, 39).

MV-induced fusion. Our negative interference experiment, showing that even at an unfavorable molar ratio, long Fus⁻ receptor molecules are dominant over functional ones, can be interpreted as indirect evidence for the existence of a MV fusion complex. In the case of influenza virus, the fusion complex may be a ring formed exclusively by HA protein trimers (13, 44). In the MV case, H-protein tetramers linked with CD46 molecules may form a scaffold around an F-protein trimer ring.

Only receptor proteins of intermediate length maintain fusion function. Interestingly, hybrid I-II/4, which is shorter than the standard receptor, is best in fusion support. This fact, and the observation that the stabilization of MV binding achieved by SCRs III and IV is dependent on the availability of MV particles (10a) lead us to speculate that after binding, the H and CD46 proteins change their conformation, apposing the viral and cellular membranes. The membrane apposition hypothesis accounts nicely for our fusion data. Long CD46 hybrids would put the cellular membrane out of reach of the F-protein fusion peptide, and coexpressed receptor molecules of different length would form irregular scaffolds incapable of supporting fusion. Certain low scaffolds, as those formed by the best fusion-supporting hybrid I-II/4, would immediately appose membranes. Influenza virus may appose membranes before fusion either by tilting the HA protein trimer (42, 43) or by pulling on the cellular membrane with a coiled-coil peptide (49).

Distance effects might influence membrane fusion during cell entry of other viruses. Transferring the HIV binding site

nearer to the cell membrane resulted in loss of fusion (35); however, this study was designed to define the CD4 domains which play a role in HIV entry and not to study a distance effect. It was also recently observed that retroviruses expressing a foreign epitope on their envelope specifically bound a new surface receptor but that infection was blocked at a post-binding step (10). It was suggested that the specific properties of the cell surface molecule chosen as an alternative receptor resulted in the release of virus cores in the wrong intracellular compartment. Our study indicates that not only the nature but also the architecture of cell surface proteins can influence the efficiency of membrane fusion, and thus of virus entry.

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