JOURNAL OF VIROLOGY, Apr. 1995, p. 2248–2256 Vol. 69, No. 4
0022-538X/95/$04.00+0

Physical Association of Moesin and CD46 as a Receptor Complex for Measles Virus

JÜRGEN SCHNEIDER-SCHAULIES,[1]* LEE M. DUNSTER,[1] REINHARD SCHWARTZ-ALBIEZ,[2] GEORG KROHNE,[3] AND VOLKER TER MEULEN[1]

Institut für Virologie und Immunbiologie, D-97078 Würzburg,[1] Deutsches Krebsforschungszentrum, D-69120 Heidelberg,[2] Elektronenmikroskopie, Theodor Boveri Institut, D-97074 Würzburg,[3] Germany

Received 18 July 1994/Accepted 22 December 1994

Recently, two cellular membrane proteins, the membrane cofactor protein CD46 and the membrane-organizing external spike protein, moesin, have been identified to be functionally associated with measles virus (MV) infectivity of cells. We investigated the functional consequences of binding of monoclonal antibodies to both molecules individually and combined on MV attachment, fusion, and plaque formation and the putative direct physical interaction of moesin and CD46. We found that antibodies to moesin or CD46 separately inhibited MV-cell interactions to a high percentage in the plaque test, by approximately 85 and 75%, respectively. The inhibition by combinations of antibodies was additive at low concentrations and complete at high concentrations. This indicates that similar sites of interaction were blocked by steric hindrance. Furthermore, antimoesin antibodies blocked the infection of CD46-negative mouse cell lines with MV. Chemical cross-linking of cell surface proteins indicated the close proximity of CD46 and moesin in the membrane of human cells, and coimmunoprecipitation of moesin with CD46 suggested their physical interaction. Immunohistochemically by electron microscopy, CD46 and moesin were found to be localized at sites of the cellular membrane where MV particles adsorbed. These data support a model of direct interaction of CD46 and moesin in the cellular membrane and suggest that this complex is functionally involved in the uptake of MV into cells.

Worldwide, measles is still a life-threatening disease, causing more than 1,000,000 human deaths per year. Measles virus (MV) is the etiologic agent of the acute disease but is responsible also for the more serious and life-threatening diseases of acute postinfectious measles encephalitis, subacute sclerosing panencephalitis, and measles inclusion body encephalitis (31). The tropism of MV is widespread, leading to infection not only of epithelial cells of the respiratory tract and peripheral blood mononuclear cells but also of endothelial cells and cells in various organs, including the central nervous system (4, 5, 11, 24, 35). MV-cell interactions interfere considerably with cellular functions of the immune and nervous system (1, 23, 31, 36) and may lead to the induction or suppression of cellular immune response genes (18, 29, 32).

Studies in the past have suggested that the cellular receptor(s) for MV is of a proteinous nature (12, 13) and might be functionally associated with the substance P receptor (7, 8) or the acetylcholine receptor (39). Recently, two cell surface molecules, CD46 (2, 25, 26) and moesin (3), have been identified to be functionally associated with MV susceptibility, the distribution of which correlates well with the widespread cellular tropism of MV (9, 10, 33). CD46 (membrane cofactor protein) is a classical transmembrane protein binding the complement proteins C3b and C4b and inhibiting complement lysis of host cells (19, 20). Transfection of mouse and hamster cells with human CD46 expression vectors enhanced the infectivity with MV by about 100-fold for certain rodent cell lines, but not others, suggesting the presence of additional factors on the cell surface and/or intracellularly which influence MV infection and replication (22, 25). The other putative receptor molecule, the membrane-organizing external spike protein, moesin, a member of the ERM (ezrin, radixin, and moesin) family of proteins (3, 28), was characterized as a heparin-binding protein which does not contain a classical transmembrane domain and is localized mainly at the inner, but also at the outer, surface of the plasma membrane (16, 17). Antibodies to moesin (3) inhibited MV attachment to intact cells, fusion, and plaque formation to an extent similar to that described for antibodies to CD46 (25, 26). However, transfection studies with moesin were not possible, since moesin was found in all tissue culture cells investigated so far. The presence of moesin in human and animal cells indicates that moesin is a good candidate to support the uptake of MV by nonhuman (CD46-negative) cells, as found for several rodent cell lines.

These independent observations demanded a direct comparative investigation of the roles of CD46 and moesin as receptors for MV and their possible functional and physical interactions. The data obtained suggest the close proximity and physical interaction of CD46 and moesin in the cellular membrane and thus the formation of a receptor complex for MV.

MATERIALS AND METHODS

Propagation of cells, MV, and antibodies. In this study we used the following cell lines from the American Type Culture Collection: the human promyelocytic-monocytic cell line U-937, the human astrocytoma cell line U-251, human HeLa cells, and the African green monkey kidney cell line Vero. These cells were cultured as previously described (29). The mouse cell lines NS20Y and L929 were grown in Dulbecco modified Eagle medium containing 5% fetal calf serum. MV strain Edmonston was propagated on Vero cells as previously described (29).

Three monoclonal antibodies (MAbs) inhibiting MV infection were obtained by screening as previously described (3). Briefly, HeLa cells were seeded into 96-well plates and grown to 80% confluency. Supernatants of cloned hybridomas from mice immunized with human U-251 cells were added to each test well and incubated at room temperature for 30 min. After incubation, MV at a multiplicity of infection (MOI) of 0.1 was added, and monolayers were incubated for 48 to 72 h at 37°C in the presence of MAbs. The hybridomas producing superna-

* Corresponding author. Mailing address: Institut für Virologie und Immunbiologie, Versbacher Strasse 7, D-97078 Würzburg, Germany. Phone: 931-2015954. Fax: 931-2013934.

tants that inhibited plaque formation were recloned, and the most effective clones were selected. The MAbs 13/42 (immunoglobulin G1 [IgG1]) and 11/88 (IgG1) recognizing CD46 and MAb 119 (IgG2a) against moesin were purified over protein G affinity columns, and stocks containing 1 mg of MAb per ml were frozen at −20°C.

The anti-CD46 antibody clone J4/48 (IgG1) was purchased from Dianova (Hamburg, Germany). The MAb 38/87 (IgG1) against moesin (15), MAb G26 against LFA-3 (IgM), MAb W6/32 (IgG2a) against human major histocompatibility complex (MHC) class I, and MAb U85 (IgG2a) not interacting with cellular proteins were produced in the laboratory. Polyclonal anti-CD46 antibodies used for electron microscopy were a gift from G. Yeh (CytoMed Inc., Cambridge, Mass.).

Rosetting assay. Rosetting assays were performed with 2×10^5 U-937 cells in 100 μl of culture medium and incubated for 1 h at 4°C with MAb (500 μg/ml) either alone or in combination. Test MAbs were antimoesin and CD46 antibodies, whilst MAbs U85 and W6/32 were used as negative controls. After the cells were washed with phosphate-buffered saline (PBS), 200 μl of MV (5×10^6 PFU/ml) was added and then incubated for 1 h at 4°C. The cells were then washed with PBS, resuspended in 200 μl of PBS containing African green monkey erythrocytes at a final concentration of 0.1%, and incubated for 1 h at 37°C. Rosettes were defined microscopically as cells with three or more erythrocytes attached to the surface.

Plaque inhibition assay. The plaque inhibition test was performed with human U-251 cell monolayers in six-well plastic dishes as previously described (3). Confluent cell monolayers (10^6 cells per well) were washed with PBS, and 100 μl of protein G-purified antibodies was added in various concentrations. After incubation at room temperature for 45 min, the monolayers were washed once with PBS, and 100 μl of PBS containing 100 PFU of MV was added to each test well. Further incubation at room temperature for 1 h was followed by a washing with PBS prior to overlaying the wells with 5 ml of Eagle minimal essential medium supplemented with 1% fetal calf serum and 1% Noble agar (Sigma). Plaques were visualized after 3 to 5 days of incubation at 37°C by a staining with neutral red.

Vaccinia virus-encoded MV-H/F cell fusion assay. To investigate the capacities of the antibodies to inhibit fusion from without, we used a vaccinia virus double recombinant encoding both MV-H and MV-F proteins (VV-H/F); (a kind gift from T. F. Wild, Lyon, France) as previously described (25, 37, 38). Briefly, cells (U-251, HeLa, and Vero) were seeded into 48-well cluster plates (10^5 per well) and grown to confluency. The monolayers were washed with PBS, and dilutions of purified MAbs (100, 50, and 10 μg/ml) in medium were added to each test well at room temperature for 30 min. VV-H/F at an MOI of 0.1 was then added and incubated for 18 h at 37°C before the cell monolayers were fixed with 3.5% paraformaldehyde in PBS and syncytium formation was observed under the microscope.

Flow cytometry. Flow cytometric analysis was performed as previously described (16). Briefly, 2×10^5 cells were incubated for 45 min on ice with 2 μg of MAb in 200 μl of fluorescence-activated cell sorter (FACS) buffer (PBS containing 0.4% bovine serum albumin [BSA] and 0.02% sodium azide). Cells were washed twice in FACS buffer and incubated with 200 μl of a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Dako) on ice for a further 45 min. After three washes with FACS buffer, flow cytometric analysis was performed on a FACScan (Becton Dickinson). For intracellular staining of epitopes, cells were fixed and permeabilized, prior to application of the first antibody, with 3.7% paraformaldehyde containing 0.25% Triton X-100 in PBS for 5 min, and unspecific binding was blocked by FACS buffer containing 10% BSA.

Electron microscopy. HeLa cells grown on coverslips were incubated with MV for 5 min at 37°C and processed for electron microscopy as previously described (6). For electron microscopical immunolocalizations, virus-treated cells were washed once with PBS and then fixed for 20 min at room temperature with 3.0% formaldehyde in PBS. Coverslips were washed twice in PBS, permeabilized by incubation for 1 to 2 min in 0.1% Triton X-100 in PBS, and washed twice for 1 min (each wash) in PBS. Nonspecific binding sites were blocked by incubation for 5 min in PBS containing 0.3% BSA. Cells were then incubated for 30 to 45 min at room temperature with a mixture of MAb 38/87 against moesin and affinity-purified polyclonal rabbit-anti CD46 (a gift from G. Yeh, CytoMed). Specimens were washed twice for 3 min (each wash) with PBS and then incubated for 1.5 h at 4°C with a mixture of secondary antibodies conjugated to 6-nm-diameter gold (anti-mouse IgG) and 12-nm-diameter gold (anti-rabbit IgG) particles (Dianova). Primary and secondary antibodies were diluted in PBS containing 0.3% BSA. Specimens were then washed, fixed, and processed for electron microscopy as previously described (6), but the staining with 0.5% uranyl acetate in distilled water was omitted.

Cross-link and immunoprecipitation of ^{125}I-labeled cell surface proteins. For labeling of cell surfaces, 2×10^7 HeLa cells were removed from the tissue culture flask nonenzymatically with calcium- and magnesium-free PBS containing 1 mM EDTA and resuspended in 500 μl of PBS. Cell surface proteins were then labeled with 0.5 mCi of $Na^{125}I$ and with the Iodo-gen iodinating reagent according to the protocol of the manufacturer (Pierce Europe, Amsterdam, The Netherlands) in the presence of the protease inhibitors AEBSF (1 mM; Calbiochem) and aprotinin (50 μM; Boehringer Mannheim).

TABLE 1. Inhibition of binding of MV (rosetting assay)[a]

Antibody (antigen)	% Rosettes	% Inhibition of rosetting
U85[b] (none)	87 ± 3	0 ± 3[c]
W6/32[b] (MHC class I)	79 ± 7	9 ± 7
38/87 (moesin)	42 ± 5	52 ± 5
119 (moesin)	29 ± 6	67 ± 5
13/42 (CD46)	36 ± 4	59 ± 4
11/88 (CD46)	36 ± 8	59 ± 8
J4/48 (CD46)	34 ± 2	61 ± 2
119 + J4/48 (moesin + CD46)	21 ± 9	76 ± 9
38/87+J4/48 (moesin + CD46)	23 ± 9	74 ± 9

[a] U-937 cells were pretreated with MAbs (500 μg/ml) either alone or in combination (see Materials and Methods). In this test system, 100% inhibition of rosette formation is usually not expected because of unspecific binding of MV and erythrocytes to cellular membranes. All tests were performed in triplicate with at least 100 cells counted, and results ± variations are given.

[b] The nonbinding MAb U85 and the MHC class I-specific MAb W6/32 were used as controls.

[c] The inhibition of MAb U85 was set to 0%, and the results of the other antibodies were normalized to this value.

For chemical cross-linking of labeled surface molecules, the 1.2-nm-long crosslinker dithiobis(succinimidylproprionate) (DSP; Pierce) was added to the cells (2×10^6 in 100 μl of PBS) in final concentrations of 2 and 5 mM for 1 h at room temperature. For immunoprecipitation, 2×10^6 labeled cells suspended in 100 μl of PBS were lysed by the addition of 1 volume of radioimmunoprecipitation assay detergent buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 0.1% sodium dodecyl sulfate [SDS], 1% sodium desoxycholate, 1% Triton X-100) in the presence of protease inhibitors, vortexed, and centrifuged at 10,000 × *g* for 10 min. Five micrograms of antibodies was added to the supernatants for 1 h on ice, and protein A-Sepharose (Pharmacia) conjugated to anti-mouse immunoglobulin (Dako) was added for 45 min. Sepharose beads were washed three times with ice-cold PBS and then dissolved in gel loading buffer for SDS-polyacrylamide gel electrophoresis (PAGE).

Western blot. Semidry Western blots (immunoblots) were performed as previously described (17). Blots were incubated with 5 μg of MAb 38/87 to moesin per ml or 5 μg of MAb 13/42 to CD46 per ml in blocking buffer (5% dry milk powder, 0.02% Tween in PBS) and then with peroxidase-conjugated anti-mouse immunoglobulin (1:100; Dako), and detection of bands was with 4-chloro-1-naphthol.

RESULTS

In order to investigate the individual functions of moesin and CD46 during MV infection, we set up a panel of experiments to determine whether both molecules act individually or in a functionally associated manner, possibly interacting physically to form a receptor complex on the surface of cells. Virus-cell interactions were first functionally investigated on three levels by the rosetting assay for virus attachment, the fusion assay (vaccinia virus-encoded MV-H/F) for cell-cell fusion, and the plaque assay for virus-cell fusion, virus-cell infection, and cell-cell fusion.

Inhibition of MV attachment by antibodies to moesin and CD46. After treatment of U-937 cells with either anti-CD46, antimoesin, or control antibodies, the attachment of MV to cells and the subsequent binding of monkey erythrocytes were measured. Cells with at least three erythrocytes bound were counted as rosettes in the microscope. Percentages of inhibition of rosette formation by single MAbs and combinations of antibodies are shown in Table 1. The antimoesin MAbs 38/87 and 119 inhibited rosette formation in the range of 50 to 70% in comparison to controls. Similar results were obtained with anti-CD46 MAbs 13/42, 11/88, and J4/48, which inhibited rosette formation by approximately 60%. The combination of antibodies to moesin and CD46 led to approximately 75% inhibition of rosette formation, suggesting that most virus-binding sites were already blocked with antibodies to either

TABLE 2. Inhibition of MV-H/F-mediated fusion (VV-H/F fusion assay)

Antibody[a] (antigen)	Inhibition[b] of fusion tested with the following cell line:		
	U-251	Vero	HeLa
K83 (MV-H)	+	+	+
U85 (none)	−	−	−
G26 (LFA-3)	−	−	−
W6/32 (MHC class I)	−	−	−
38/87 (moesin)	+	+	+
119 (moesin)	+	+	+
13/42 (CD46)	+	+	+
11/88 (CD46)	+	+	+
J4/48 (CD46)	+	−[c]	+

[a] The neutralizing MAb K83 recognizing MV-H was used as a positive control for inhibition of fusion to cell surface molecules. The MAbs U85, G26, and W6/32 do not inhibit fusion and were used as negative controls.

[b] +, inhibition of fusion; −, no inhibition of fusion. All tests were performed in triplicates.

[c] The MAb J4/48 is specific for the human CD46 molecule and does not recognize the CD46 homolog on the African green monkey cell line Vero.

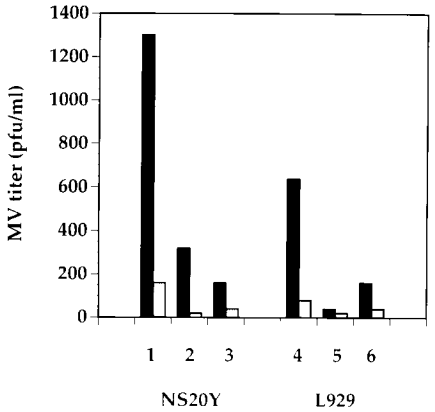

FIG. 2. Inhibition of MV yield by antibodies to moesin in susceptible mouse cell lines. The mouse cell lines NS20Y (lanes 1 to 3) and L929 (lanes 4 to 6) were infected with MV (MOI = 1) for 1 h at 37°C either without pretreatment (lanes 1 and 4) or after pretreatment with 75 μg of antibodies to moesin (MAb 38/87, lanes 2 and 5, or MAb 119, lanes 3 and 6) per ml for 1 h at room temperature. After 48 h of incubation at 37°C, the cell-bound (solid bars) and supernatant (shaded bars) MVs were titrated on Vero cells. Both antibodies to moesin considerably reduced the yield of infectious MV.

molecule and that only a small percentage of binding sites was independently used (Table 1).

Inhibition of cell fusion by antibodies to moesin and CD46. We tested the cell-cell fusion (fusion from without) mediated by recombinant MV-H and -F proteins together expressed by vaccinia virus (VV-H/F) (25, 37, 38). The results achieved in this test system are presented in Table 2. Antibodies to moesin or CD46 (dilutions of 100, 50, and 10 μg/ml) alone inhibited the capacity of recombinant MV-H and -F proteins to mediate cell fusion completely, whereas unrelated antibodies binding to other cell surface structures as controls (MHC class I and LFA-3) did not abolish fusion. Similar results were achieved with the human cell lines HeLa and U-251 and with African green monkey Vero cells, with the exception of MAb J4/48, which does not recognize the monkey CD46 homolog and consequently did not inhibit VV-H/F fusion on these cells. The MV-H-specific MAb K83 was used as a positive control, inhibiting fusion by interaction with the viral hemagglutinin. Since antibodies to moesin or CD46 alone inhibited fusion so effectively, we did not test combinations of antibodies in this assay system.

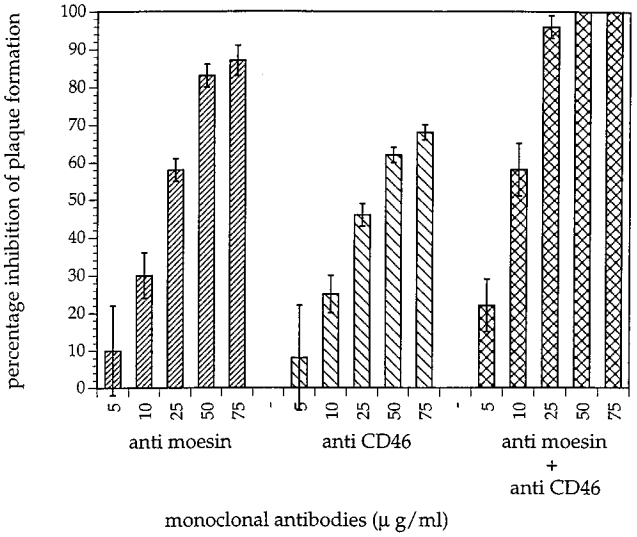

FIG. 1. Inhibition of plaque formation by MAbs against CD46 and moesin. Monolayers of human astrocytoma cell line U-251 were incubated with increasing concentrations of MAbs either alone or in combination as indicated. After washing the monolayers, cells were infected with 100 PFU of MV and proceeded as for regular plaque assay (see Material and Methods). Results of three experiments were averaged and are given as percentages of inhibition of plaque formation ± standard deviations.

Inhibition of plaque formation by antibodies to moesin and CD46. The plaque test assays viral infectivity on the level of attachment, virus-cell fusion, and cell-cell fusion (fusion from within and from without). Interaction of antibodies with target cells leading to inhibition of plaque formation was investigated in a dose-dependent manner. Monolayers of HeLa cells were first treated with antibodies and washed prior to incubation with 100 PFU of MV per dish. Antibodies to moesin (MAb 119) and CD46 (MAb J4/48) were applied in concentrations of 5 to 75 μg/ml either alone or in combination (Fig. 1). High concentrations of single MAbs inhibited plaque formation maximally by approximately 85% (MAb 119) and 70% (MAb J4/48). Combinations of MAbs at low concentrations (5 and 10 μg/ml) led to a percentage of inhibition approximately additive of the effects of single antibodies. Higher concentrations of MAbs in combination, thus saturating MV-binding sites, led to complete inhibition of plaque formation (Fig. 1). Similar results were obtained with MAb 38/87 against moesin, inhibiting by maximally 55% (3), and with Fab fragments of MAb 119 against moesin and MAb 13/42 against CD46 (not shown).

MAbs recognizing other strongly expressed cell surface molecules were used as controls. The MAbs W6/32 (MHC class I) and G26 (LFA-3) inhibited maximally by 15 and 5%, respectively. These percentages of inhibition are likely to result because antibodies against these highly expressed molecules cover large parts of the cell surface and were considered to result from nonspecific inhibition of MV binding.

Antimoesin antibodies inhibit the infection of mouse cells. Some mouse cell lines which do not express CD46 can be productively infected with MV. It has been described that transfection of such cells with expression vectors for CD46 enhanced the production of infectious MV by approximately 100-fold (22, 25). Since our antibodies against moesin reacted with the surface of all mouse cells tested (not shown), we

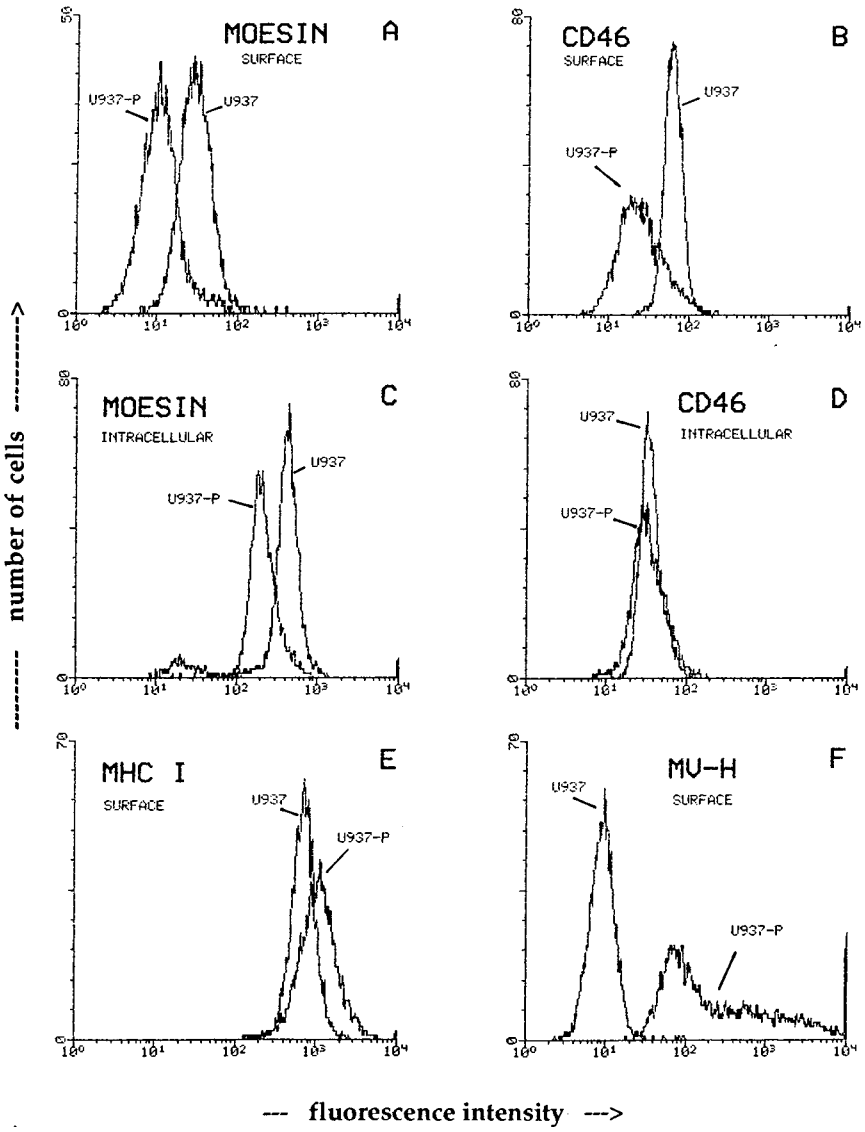

FIG. 3. Flow cytometric analysis of surface and intracellular expression of moesin and CD46 by U-937 and persistently MV-infected U-937-P cells. Cells were processed for surface staining (A, B, E, and F) or permeabilized for intracellular staining (B and D). Cells were stained with antimoesin MAb 38/87 (A and C), anti-CD46 MAb 13/42 (B and D), anti-MHC class I MAb W6/32 (E), and anti-MV hemagglutinin K83 as a control for infection (F) and fluorescein isothiocyanate-conjugated second antibodies. Downregulation of moesin in persistently infected cells is observed from the surface and intracellularly (A and C), whereas downregulation of CD46 is observed exclusively from the surface (B) and not intracellularly (D).

investigated whether moesin is involved in the uptake of MV as observed in the absence of CD46. The susceptible mouse cell lines NS20Y and L929 were pretreated for 1 h at room temperature with the antimoesin MAbs 38/87 and 119 or with PBS as a control, followed by infection of the cells with MV (MOI = 1). After 24, 48, and 72 h at 37°C, the titers of infectious MV in the cell associated and supernatant fraction of both cell lines were determined. Maximal titers were obtained after 48 h (Fig. 2). Antibodies to moesin reduced the titer of MV in NS20Y cells by approximately 80% and that in L929 by approximately 85%. These data suggest that moesin is functionally involved in the uptake of MV by mouse cells which do not express CD46.

Expression of moesin and CD46 on the cell surface of human cells during infection. Since the three functional assays described above with the human cell lines suggested an associated action of CD46 and moesin, we investigated the localization and interaction of both molecules in human cells at the molecular level. Whereas CD46 is a class I transmembrane protein exposing its amino terminus on the outside of the cell, moesin does not contain a classical transmembrane domain, and its orientation in or on the membrane is not known. Nevertheless, moesin not only is expressed intracellularly but also was detected on the surface of cells by our antibodies (Fig. 3A). When we compared the two extremes of uninfected and persistently MV-infected cells, moesin was downregulated from the surface of persistently infected U-937 cells (Fig. 3A). CD46 was highly expressed on the surface of most human tissue culture cells and downregulated from the surface after persistent infection with MV (Fig. 3B). A greater amount of moesin was detected intracellularly compared with that detected extracellularly in U-937 cells, and downregulation was also observed for intracellular expression of moesin in persis-

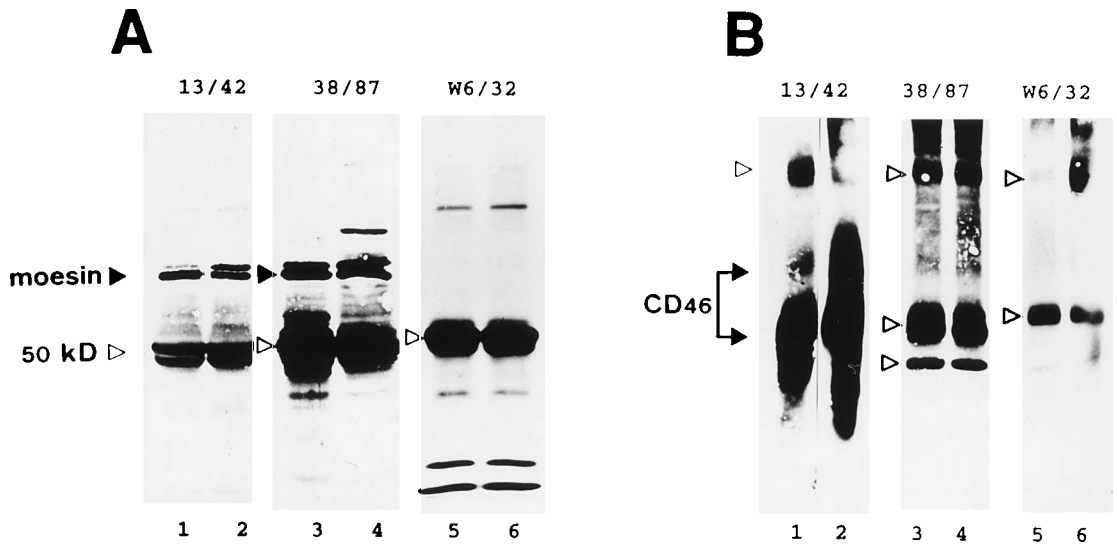

FIG. 4. Immunoprecipitation of moesin and CD46 from extracts of U-937 and HeLa cells and consecutive detection of precipitated molecules by Western blot. Proteins in extracts of U-937 (lanes 1, 3, and 5) and HeLa cells (lanes 2, 4, and 6) were precipitated with MAb 13/42 against CD46 (lanes 1 and 2), MAb 38/87 against moesin (lanes 3 and 4), and MAb W6/39 against MHC class I (lanes 5 and 6) as indicated at the top. Proteins were separated on 10% polyacrylamide gels under reducing (A) or nonreducing (B) conditions and blotted on nitrocellulose. The Western blots were developed with antibodies against moesin (A) and CD46 (B). Specific bands of moesin at 75 kDa (A, solid triangles) were detected not only in material precipitated with antibodies to moesin (A, lanes 3 and 4) but also in material precipitated with antibodies to CD46 (A, lanes 1 and 2). Bands at 77 kDa (A, lanes 1 to 4) could represent isoforms of moesin or the protein radixin, and the band at 90 kDa (A, lane 4) is an unknown cross-reacting protein. No moesin band was detected in proteins precipitated with antibodies against MHC class I (A, lanes 5 and 6). Bands at 50 kDa represent the heavy chains of antibodies used for immunoprecipitation and detection with the peroxidase-conjugated second antibodies on the Western blot (A, open triangles). Specific bands of CD46 were detected after precipitation with MAbs against CD46 (B, lanes 1 and 2, solid triangles) and not with MAbs to moesin and MHC class I (B, lanes 3 to 6). Other detected bands are caused by cross-reacting antibodies used for immunoprecipitation (B, open triangles). (B) This gel was run under nonreducing conditions.

tently MV-infected cells (Fig. 3C). In contrast, the expression of CD46 in permeabilized U-937 cells was not affected by the infection of U-937 cells with MV (Fig. 3D). Stainings of the cells with antibodies against MHC class I (Fig. 3E) and MV hemagglutinin (Fig. 3F) is shown as controls. These results show that a percentage of moesin is present at the cell surface and that the surface expression of both moesin and CD46 is affected by MV infection.

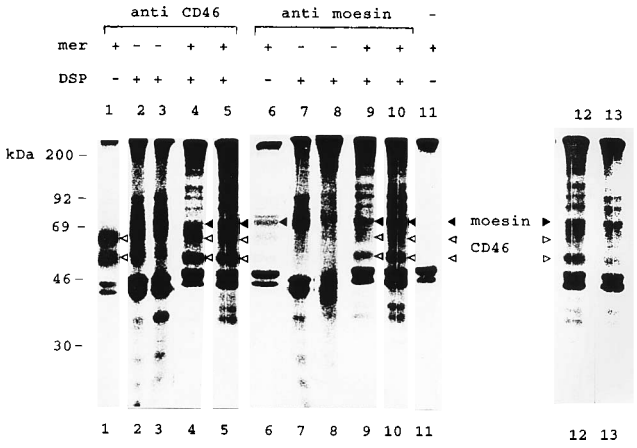

FIG. 5. Chemical cross-link of moesin and CD46 with the cleavable cross-linker DSP. HeLa cell surface proteins were labeled with $Na^{125}I$. After cross-linking with DSP, CD46- and moesin-containing complexes were immunoprecipitated with anti-CD46 (lanes 1 to 5) and antimoesin (lanes 6 to 10) MAbs but not the nonbinding MAb U85 (lane 11). Lanes 1 and 6, immunoprecipitated material without cross-linker; lanes 2 and 3 and 7 and 8, cross-linked material with 1 and 5 mM DSP, respectively, without cleavage; lanes 4 and 5 and 9 and 10, cross-linked material with 1 and 5 mM DSP, respectively, cleaved in the presence of 100 mM β-mercaptoethanol; lane 11, control precipitation with nonspecific antibody. Bands in the molecular weight ranges of moesin (closed triangles) and CD46 (open triangles) were detected in precipitated material with antibodies to moesin as well as CD46. Lanes 12 and 13, less intensive exposures of lanes 5 and 10, respectively. Background bands present in all lanes at 43 kDa are probably actin.

Coimmunoprecipitation of moesin and CD46. To investigate whether the immunoprecipitates obtained with antibodies against moesin or CD46 contained the other molecule, we probed proteins immunoprecipitated with antibodies against one molecule on Western blots with antibodies to the other molecule. The immunoprecipitated material from U-937 and HeLa cells with antibodies to CD46 (13/42), moesin (38/87), and MHC class I (W6/32) was separated by SDS-PAGE and blotted on nitrocellulose, and the Western blots were developed with the antimoesin MAb 38/87 (Fig. 4A) and the anti-CD46 MAb 13/42 (Fig. 4B).

Clear bands representing moesin were detected in the extracts precipitated with antibodies against moesin as well as CD46 (Fig. 4A, lanes 1 to 4) but not with antibodies against MHC class I (Fig. 4A, lanes 5 and 6). The proteins immunoprecipitated with antibodies to moesin (Fig. 4A, lanes 3 and 4) contained large amounts of the specific moesin band at 75 kDa and a band at approximately 77 kDa, the intensity of which depends on the cell line investigated. Similar, but less intensive, bands were observed in the anti-CD46-precipitated material (Fig. 4A, lanes 1 and 2). The broad bands at 50 kDa represent the heavy chains of the antibodies used for immunoprecipitation, which were detected by the second antibody used in the Western blot.

The SDS-PAGE for the Western blot developed with antibodies against CD46 (Fig. 4B) was performed under nonreducing conditions because of the dependence of the recognition of CD46 by MAbs on conformation (20, 21, 27). In this case, large amounts of CD46 (broad bands) could be detected in material which was immunoprecipitated with antibodies to

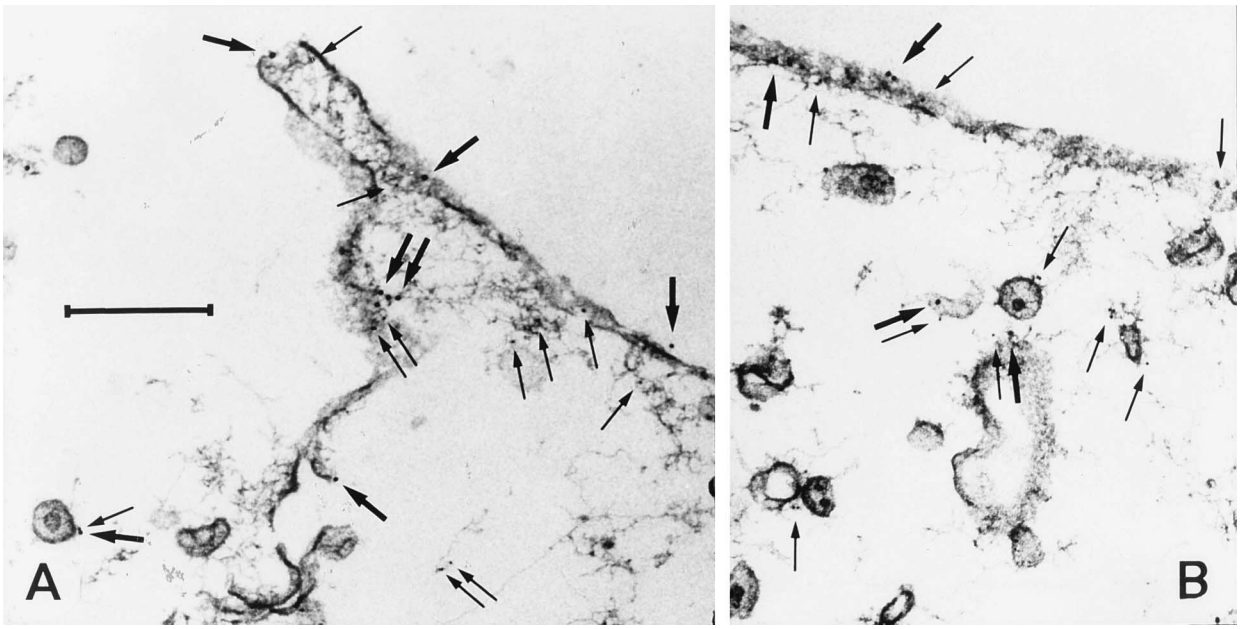

FIG. 6. Electron microscopic localization of moesin and CD46 in noninfected HeLa cells. HeLa cells were fixed and permeabilized and processed for electron microscopy (see Materials and Methods). Moesin and CD46 were visualized after treatment with MAb 38/87 against moesin and polyclonal antibodies against CD46 and secondary gold-conjugated antibodies (anti-mouse IgG, 6-nm-diameter gold particle, and anti-rabbit immunoglobulin, 12-nm-diameter gold particle). (A and B) Thin arrows indicate moesin, and larger arrows indicate CD46. (A) Bar, 100 nm.

CD46 (Fig. 4B, lanes 1 and 2), but only very little was detected in material immunoprecipitated with antibodies to moesin (Fig. 4B, lanes 3 and 4). No signal was obtained in material immunoprecipitated with antibodies to MHC class I (Fig. 4B, lanes 5 and 6). Some reasons for not detecting CD46 in anti-moesin-precipitated material could be that the diffuse bands of CD46 do not reach the intensity required for detection and that the lower-molecular-mass CD46 species is in a range of cross-reacting bands of the antibodies used for immunoprecipitation. Thus, it was possible to demonstrate direct coimmunoprecipitation in one particular manner, namely, moesin coprecipitated with antibodies against CD46, whereas CD46 coprecipitation with antibodies against moesin could not clearly be demonstrated.

Chemical cross-link of moesin and CD46. Since CD46 was hardly detectable on the Western blot in antimoesin-precipitated material, we performed cross-linking studies with chemical cross-linkers on the surface of native HeLa cells labeled with ^{125}I to test whether both molecules are located in proximity at the cell membrane. Best results were obtained with the cleavable cross-linker DSP (see Materials and Methods). Cell extracts were immunoprecipitated with antibodies to CD46 (Fig. 5, lanes 1 to 5), antibodies to moesin (Fig. 5, lanes 6 to 10), and nonbinding control antibody (Fig. 5, lane 11). Cell extracts without cross-linker were precipitated as controls (Fig. 5, lanes 1 and 6) and with 2 and 5 mM DSP (Fig. 5, lanes 2 and 3, 4 and 5, 7 and 8, and 9 and 10, respectively). The cross-linked complexes were either left intact under nonreducing conditions (Fig. 5, lanes 2, 3, 7, and 8) or separated by cleavage of the cross-linker in the presence of 100 mM β-mercaptoethanol (Fig. 5, lanes 4, 5, 9, and 10). Bands of the apparent molecular mass of moesin and CD46 were detected in cleaved complexes precipitated with antibodies to CD46 as well as moesin. Less intensive exposures of lanes 5 and 10 are shown in lanes 12 and 13. This experiment demonstrated that CD46 and moesin are expressed in close proximity on the surface of uninfected HeLa cells.

Immunohistochemical localization of moesin and CD46. Electron microscopical analyses of HeLa cells with double staining of CD46 and moesin with antibodies and gold-conjugated second antibodies were undertaken to visualize the presence of both molecules in the membrane of uninfected cells and at sites of MV-cell interaction. In uninfected cells (Fig. 6), CD46 and moesin were found to be expressed mainly at membranes at the cell surface and inside the cell. Moesin (Fig. 6, 6-nm-diameter gold particles) and CD46 (Fig. 6, 12-nm-diameter gold particles) were found individually, as well as in close proximity.

For cells which were exposed for 5 min to MV, electron-dense MV particles were found to be adsorbed predominantly near microvilli (Fig. 7A and B). These microvilli were also described to be the sites of the highest-level moesin expression (28). After double staining of such cells with antibodies to CD46 and moesin in conjunction with gold-conjugated second antibodies, clusters of moesin and CD46 were observed in the vicinity of MV particles (Fig. 7C and D). The large arrow in Fig. 7C indicates an MV particle attached to the cell membrane. The enlargement (Fig. 7D) shows the presence of several 6- and 12-nm-diameter gold grains, indicating moesin and CD46 at this site of MV attachment. In most cases MV particles were observed in the proximity of at least one type of gold particles, and both types of grains were present at approximately 70% (not shown). Thus, CD46 and moesin are found in close proximity at sites where MV particles attached to the cell surface.

DISCUSSION

The independent findings that antibodies against CD46 and moesin can inhibit infection of cells with MV led us to investigate the interaction of these cellular membrane molecules. In our approach we wanted to answer the question of whether both molecules possibly act (i) independently as alternative receptors or (ii) in an associated manner. According to model

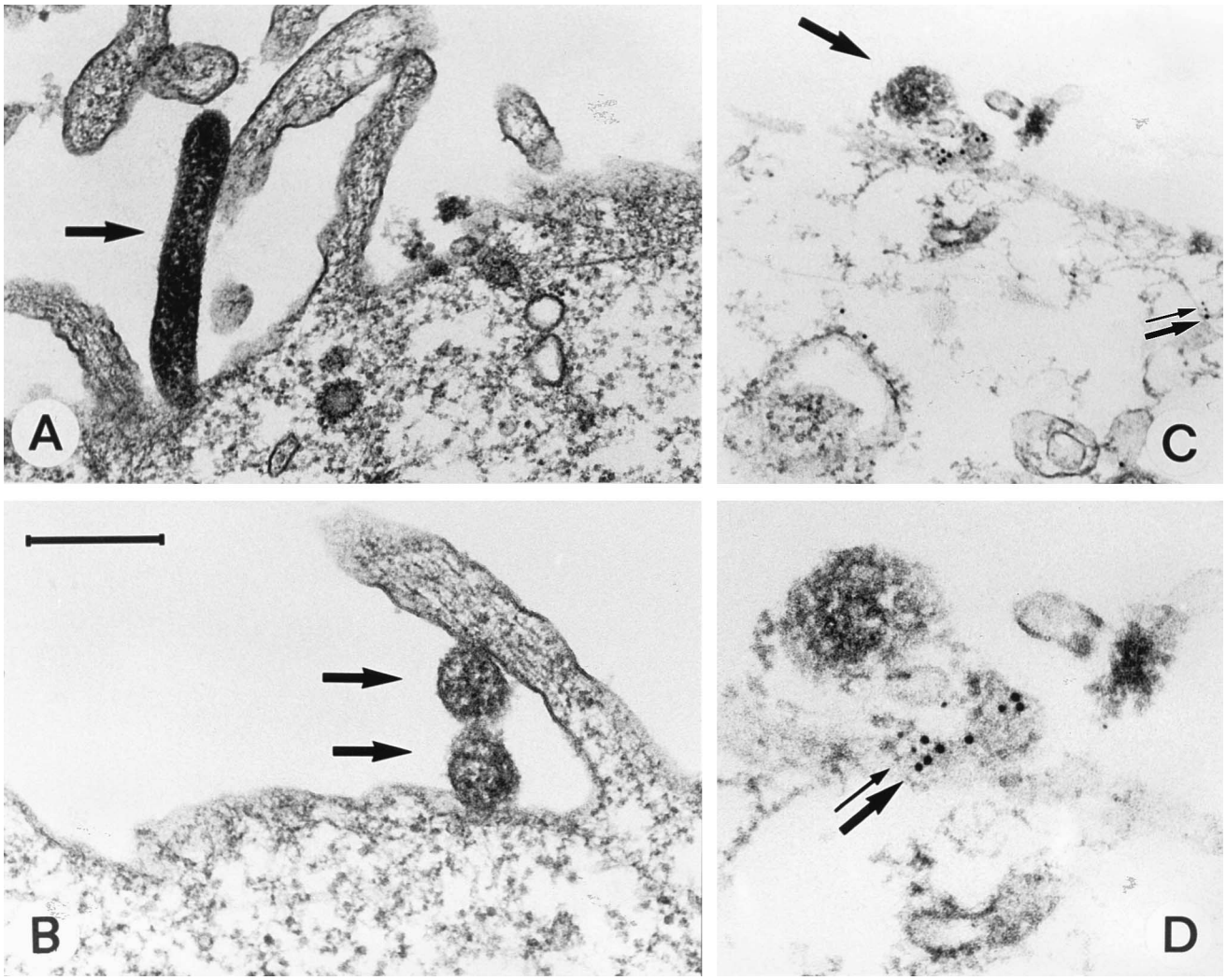

FIG. 7. Electron microscopic localization of MV particles, moesin, and CD46. MV was exposed to HeLa cells for 5 min at 37°C, and coverslips were processed for electron microscopy. MV particles are the dark, electron-dense lengthy particles (arrows in panels A and B) adsorbing predominantly at the basis of cellular microvilli. MV-adsorbed HeLa cells were treated with MAb 38/87 against moesin and polyclonal antibodies against CD46 and secondary gold-conjugated antibodies (anti-mouse IgG, 6-nm-diameter gold particles, and anti-rabbit immunoglobulin, 12-nm-diameter gold particles C and D). The large arrow in panel C indicates an MV particle, and the pair of arrows inside the cell point to a pair of CD46 and moesin. The MV particle is located in close proximity to a cluster of moesin and CD46 as shown in panel C and enlarged in panel D (small and large arrows, respectively). (B) Bar, 100 nm. The same enlargement is shown in panels A, B, and C, whereas panel D is a 3.2-fold enlargement from panel C.

i of independent uptake mechanisms of MV into cells with alternative receptors, antibodies against both molecules alone should inhibit by only low percentages, since exclusion of one receptor would still leave the second available for interaction. In combination at higher concentrations, antibodies should inhibit additively, with the sum of the individual percentages of inhibition maximally adding up to 100%. This is not the case with human cells, since individual antibodies to CD46 and moesin already inhibited attachment, fusion, and plaque formation by more than 50%. However, in the case of mouse cells, our data suggest that moesin provides the means by which MV can enter cells in the absence of CD46 (Fig. 2). The level of MV uptake mediated by moesin in the absence of CD46 is considerably less than found in the presence of CD46, which has been shown by transfection of mouse cells with CD46 expression vectors (22, 25). Our data do not exclude that moesin on mouse cells is associated with an as yet unknown molecule contributing to the uptake of MV in these cells. The antibody 38/87 against moesin (75 kDa) used in this study also detected a 77-kDa band on the Western blot (Fig. 4A). This upper band could be either an isoform of moesin or the highly homologous cross-reacting protein radixin, a member of the ERM (ezrin, radixin, and moesin) family of proteins (28). The affinity of CD46 for moesin and this upper band seems to be similar to the affinity of the MAb 38/87 for the two proteins, since the ratio of the intensity of the bands is similar in the anti-CD46 (Fig. 4A, lanes 1 and 2)- and antimoesin (Fig. 4A, lanes 3 and 4)- precipitated material.

According to model ii of an uptake mechanism with CD46 and moesin acting in association, the addition of single MAbs to the complex should inhibit to high percentages and the combination of MAbs should inhibit additively at low antibody concentrations. Our results regarding the functional assays with human cells support this second hypothesis with an associated uptake mechanism of MV. The fact that the initial step of MV infection, namely, adsorption to the cell surface, was

inhibited by 60 to 70% by the addition of single MAbs and not much more by the addition of combinations of MAbs suggests that MV-binding sites are sterically overlapping with binding sites of the MAbs to both moesin and CD46. Plaque formation was inhibited maximally by 85% with MAbs against moesin alone and 70% with MAb J4/48 against CD46 alone. This strongly supports the model of associated molecules, as both MAbs directed against a single molecule were responsible for more than a 50% reduction in MV infectivity. Furthermore, the results of these biological assays with living cells show that moesin, which has been supposed to be located at the inner side of the cell membrane, is also recognized by antibodies to a certain extent at the outside of cells.

In contrast to results described by Dörig et al. (2), we found that MAb J4/48 inhibited MV strain Edmonston attachment, fusion, and plaque formation on human cells. Similar results were obtained with our MAb 13/42, which also reacted with the African green monkey (Vero) CD46 homolog. These differences probably reflect the use of alternative protocols in the rosetting assay, which we have performed with cells in suspension, and the syncytium formation assay, in which we used a 100-PFU test. A further explanation for the difference in results may concern the MV strain Edmonston used by Dörig et al. (2) and that used in our experiments.

Inhibition of MV binding by steric hindrance requires the close proximity (at least transient) of both molecules at the cell surface. Our studies with chemical cross-linking of cell surface molecules of uninfected living cells support the hypothesis that CD46 and moesin are located in close proximity, within 1.2 nm, at the cell membrane. Furthermore, the coimmunoprecipitation of moesin with antibodies to CD46 without involvement of cross-linkers indicates a physical interaction between both molecules. The converse experiment did not lead to clearly visible bands of CD46 coimmunoprecipitated with antibodies against moesin. The reason for this is probably that the CD46 bands are diffuse and therefore require larger amounts for detection than the moesin band. In addition, cross-reacting antibody bands are present in the same region of the blot. In contrast, small amounts of moesin can be detected on Western blots, since this protein migrates with a concentrated band in SDS-PAGE and the affinity of the MAb 38/87 is very high. According to the immunohistochemical localization, moesin and CD46 are present at microvilli, sites where the adsorption of MV particles is predominantly observed. The immunogold-labeled antibodies demonstrate the presence of clusters of moesin and CD46 in association with MV particles adsorbed to the cell membrane. In the light of our results of the functional assays, we interpret these data as CD46 and moesin forming a complex supporting MV infection of cells.

This interaction may take place between only a fraction of CD46 and moesin present at the cell surface and does not exclude that both molecules might react intracellularly also with other partners. In the case of moesin, an association with cytoskeleton proteins like actin, which could possibly provide a mechanism for subsequent steps for MV uptake into cells and replication, has been suggested (28). The interaction with other molecules might also explain differences observed for intracellular expression of moesin and CD46. CD46 is present at the cell surface for at least 3 days, since antibody (MAb 13/42)-treated cells remain resistent to MV infection, and the presence of the antibody on the cell surface can be shown by interaction with secondary antibodies (unpublished results). Moesin is easily cleaved by trypsin, and its stability and turnover rate might be different from those of CD46 and must be further investigated.

Evidence that more than one cell surface molecule might generally be involved in mediating susceptibility of cells to viruses has been accumulated. Either second receptors or functionally associated molecules are necessary for efficient infection of cells. Recently, for poliovirus an MAb which can inhibit infection and is directed against CD44, a molecule which does not directly bind to poliovirus, was described (34). The mechanism of interference with receptor binding and infection of cells is not clear in this case. For MV, direct binding has until now been demonstrated only to CD46 (21) and not to moesin. However, the infection of CD46-negative rodent cells at low levels, which is inhibitable by antibodies against moesin, suggests that moesin alone is able to support the uptake of MV and therefore suggests direct binding of MV also to moesin. Since transfection of such mouse cells with CD46 expression vectors enhanced the infectivity by about 100-fold (22, 25), we suppose that this is due to a higher-affinity binding of MV hemagglutinin to CD46. It is not known which exact function moesin might provide in the complex with CD46 for MV uptake into cells. These findings may be cell type specific, with the level of expression of moesin and CD46 influencing the rate and quantity of MV uptake. The roles of CD46 and moesin for the entry mechanism of MV into cells as well as MV strain variations are currently under investigation.

ACKNOWLEDGMENTS

We thank S. Löffler and K. Pech for excellent technical assistance and G. Yeh (CytoMed) for providing us with the polyclonal anti-CD46 antiserum used in this study.

This work was supported by the Deutsche Forschungsgemeinschaft and Bundesministerium für Forschung und Technologie.

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