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CD9 clustering and formation of microvilli zippers between contacting cells regulates virus-induced cell fusion

Katrin Singethan¹, Nora Müller¹, Sabine Schubert¹, Doreen Lüttge¹, Dmitry N Krementsov², Sandhya R Khurana², Georg Krohne³, Sibylle Schneider-Schaulies¹, Markus Thali², and Jürgen Schneider-Schaulies^{1,*}

¹Institute for Virology and Immunobiology, University of Würzburg, Versbacher Str. 7, D-97078 Würzburg, Germany

²College of Medicine and CALS, University of Vermont, Department of Microbiology and Molecular Genetics, Burlington, VT 05405-0084, USA

³Division of Electron Microscopy, Biocenter, University of Würzburg, Am Hubland, D-97070 Würzburg, Germany

Abstract

Members of the tetraspanin family including CD9 contribute to the structural organization and plasticity of the plasma membrane. K41, a CD9-specific mAb, inhibits the release of human immunodeficiency virus (HIV-1), and canine distemper virus (CDV)-, but not measles virus (MV)-induced cell-cell fusion. We now report that K41, which recognizes a conformational epitope on the large extracellular loop (LEL) of CD9, induces rapid relocation and clustering of CD9 in net-like structures at cell-cell contact areas. High resolution analyses revealed that CD9 clustering is accompanied by the formation of microvilli that protrude from either side of adjacent cell surfaces, thus forming structures like microvilli zippers. While the cellular CD9-associated proteins β 1-integrin and EWI-F were co-clustered with CD9 at cell-cell interfaces, viral proteins in infected cells were differentially affected. MV envelope proteins were detected within, whereas CDV proteins were excluded from CD9 clusters. Thus, the tetraspanin CD9 can regulate cell-cell fusion by controlling the access of the fusion machinery to cell contact areas.

Keywords

tetraspanin; CD9 clustering; microvilli; cell fusion; canine distemper virus (CDV); measles virus (MV)

INTRODUCTION

Laterally interacting tetraspanins in the plasma membrane together with other cell membrane receptors form tetraspanin-enriched microdomains (TEMs), also called the tetraspanin web (1-5). Within TEMs, CD9 interacts with β 1-integrins, pro-HB-EGF, EWI-2, EWI-F, FPRP, PSG17, and other cell surface proteins (6-10), regulates the plasticity of the cellular membrane, and influences the cell motility and cell fusion (4,11-16). In addition, CD9 modulates also various virus-induced processes at membranes such as membrane fusion, viral budding and release (17-23).

*Corresponding author. Tel: +49-931-20149895; FAX: +49-931-20149553, jss@vim.uni-wuerzburg.de.

Online supplementary material. 2 movies, 2 figures.

Another tetraspanin, CD63, was found to be incorporated into HIV particles (24-26) and to colocalize with CD9 and HIV-1 Gag-proteins in TEMs on the cell surface (23). Treatment of cells with mAb K41 induced the clustering of CD9 and also other tetraspanins (CD63, CD81) and inhibited HIV-1 release suggesting that normal non-aggregated tetraspanin-containing microdomains may be important for egress of the virus. Interestingly, the release of another enveloped virus, influenza virus, was not affected by disruption of TEMs by mAb K41 (27). In the case of canine distemper virus (CDV), mAb K41 inhibits the virus-induced cell-cell fusion and virus release, whereas virus uptake is not affected (20,21). The extracellular domain of the viral haemagglutinin (H) of CDV determines the susceptibility of the cell-cell fusion to certain CD9-antibodies, however does not itself bind to CD9 (28). This suggested that structural alterations of the plasma membrane influencing the activity and/or spatial expression pattern of receptors are involved.

The nature and consequences of antibody-induced CD9 clustering on membrane plasticity and virus-induced cell fusion remained unresolved. We found that K41 induces characteristic CD9 clusters exclusively at cell-cell contact areas, and that antibody-induced CD9-aggregation is associated with the formation of CD9-enriched microvilli-like protrusions. CD9 binding partners such as EWI-F are co-clustered into these cell-cell contact areas, whereas proteins of specific viruses such as CDV are displaced. The exclusion of the viral fusion machinery from CD9 clusters and its physical separation from cell contact areas provides an explanation for the inhibition of virus-induced cell-cell fusion by K41.

RESULTS

Complete bivalent mAb K41, but not Fab fragments, induces CD9 clustering at cell-cell contact areas

In untreated epithelial and endothelial cells, CD9 is evenly distributed over the cell surface with denser appearing lines of CD9 at the cell edges (Fig. 1). Treatment of cells for 20 h with 5-15 µg/ml mAb K41 induced large, strongly CD9-positive aggregates. These clusters emerged exclusively at cell surface areas in contact with neighboring cells (cell-cell contact areas), but not at parts of the plasma membrane facing medium or the plastic dish. Similar CD9 clusters were observed on epithelial (Vero, HeLa) and endothelial (HUVEC) cells with cell specific variations in size and density (Fig. 1 A-F). On HeLa cells these aggregates appear very dense reaching a length of up to 10 µm, on Vero cells up to 20 µm and on HUVEC, expressing the highest levels of CD9 (shown in Fig. 2 A), up to approximately 30 µm. When signal intensities of micrographs were reduced to resolve structures in these CD9 clusters (Fig. 1 B, D, F), non-aggregated CD9 is not detected due to the lower fluorescence intensity. However, CD9 does not completely disappear from the cell body and can be visualized when signal strength is enhanced. Only certain CD9-specific mAbs induced this phenomenon (see below). Various antibodies to other tetraspanin family members such as CD63 and CD81 did not induce CD9 clustering (not shown), or corresponding clustering of the homologous tetraspanins they are recognizing (Fig. 1 G-J).

To analyze which of the two extracellular domains of CD9 is recognized by mAb K41, we used expression plasmids of chimeric tetraspanins in which CD9, CD81 and CD82 contributed different parts, as indicated (Fig. 1 K). CD9-negative CHO cells were transfected and 48 h later stained with mAb K41 and secondary antibodies to quantify antibody binding by flow cytometry. MAb K41 specifically recognizes the large extracellular loop (LEL) of CD9 (Fig. 1 K). In addition, the epitope recognized by K41 is sensitive to reducing agents indicating that the disulfide bridges present in the LEL of CD9 are important to stabilize the conformational epitope recognized by K41.

We investigated whether Fab fragments of mAb K41 (IgG1 κ) may have the same effect on CD9 as the complete bivalent antibody. In immunofluorescence control experiments Fab fragments recognized CD9 similarly as the complete bivalent mAb K41 (Fig. 2 A). However, incubation of cells with 2-5 μ g/ml Fab fragments, which provides the same molarity of epitope binding sites as 5-15 μ g/ml complete K41, did not result in CD9 clustering, and even incubation of cells with 10 μ g/ml Fab fragments did not induce CD9 aggregation. In addition, crosslinking the Fab fragments with secondary antibodies also resulted in only marginal CD9 clustering (not shown). Corresponding results were obtained with respect to the capacity of the antibodies to inhibit the CDV-induced cell-cell fusion. Fab fragments alone or in combination with crosslinking secondary antibodies did not inhibit the cell-cell fusion in cells transfected with expression vectors of CDV envelope proteins haemagglutinin (H) and fusion protein (F) (Fig. 2 B). Thus, CD9 clustering is induced only with the complete bivalent antibody.

Kinetics of CD9 clustering

In order to examine the CD9 relocation and clustering in more detail in live cells, we used HeLa cells stably expressing a CD9-eGFP fusion protein. These cells moderately (approximately three times) overexpress CD9 in comparison to parental HeLa cells. Clustering of CD9 at cell-cell contact sites after 0 - 6 h treatment of cells with 10 μ g/ml mAb K41 is shown in Fig. 3A and two movies. Fig. 3A documents that significant CD9 clustering was already observed 30 min after addition of K41 and large clusters were detected after 3 h (arrow). Timelapse analyses of K41-treated HeLa-CD9-eGFP cells are presented in two movies, one of which spans the first hour with high time resolution (1 picture/min), and the other 6 h with lower time resolution (1 picture/15min; supplementary material www://...). In addition, we addressed the question whether the CD9 aggregates are composed of relocated CD9, or if the cells require higher amounts of CD9. Quantifications by Western blots of the steady state levels of CD9 in Vero, HeLa, and endothelial cells 0, 2, 6, and 20 h after treatment with K41 demonstrated that there is no significant increase of total cellular CD9 (Fig. 3 B). These data indicate that CD9 present on the cell surface or in intracellular stores is rapidly relocated to cell-cell contact sites.

High resolution analyses of CD9 clusters reveal microvilli-like protrusions

CD9 clusters at cell contact areas were further investigated at higher resolution using laser scanning (Fig. 4) and scanning electron microscopy (SEM; Fig. 5). By confocal microscopy analysis CD9 clusters appeared to be net-like structures located exclusively between contacting cells, but neither at parts of the membrane where cells are in contact with the culture dish, or on free surfaces. Similar structures were observed on interacting Vero, HeLa and HUVEC (Fig. 4 B, G, I). A three-dimensional reconstruction of the CD9 aggregates showed that these net-like structures consist of globular CD9-positive membrane protrusions (Fig. 4 C). When F-actin (green) was visualized in addition to CD9 (red), actin fibers could be detected in close association with these globular structures, but were not present within CD9 clusters (Fig. 4 E). When the actin cytoskeleton was disrupted with latrunculin B, CD9 net-like structures were not formed (supplementary Fig. 1). In addition we tested inhibitors of small the GTPases Rho/Rac/CDC42 (lethal toxin of *Clostridium sordellii*, toxin B of *Clostridium difficile*, Y-27632, Rac1-inhibitor), a PI3-kinase inhibitor (up to 1 mM), a PI4-kinase inhibitor (phenyl oxide, up to 100 nM), a PKA inhibitor (adenosine-3',5'-cyclic monophospho-phoro thioate, up to 200 μ M), and a PKC inhibitor (Ro-32-0432, up to 550 nM), which all did not inhibit the formation of net-like structures in response to K41 treatment (not shown).

Aggregates comparable to the ones induced by mAb K41 were generated also by incubation with other CD9-antibodies such as clone P1/33/2 (Fig. 4 J, K), MM2/57 (Abcam), and

ALB-6 (Abcam), but not for example by incubation with clone H110 (Fig. 4 L, M, and (27)). All antibodies inducing the formation of CD9 clustering also inhibited the CDV-induced cell fusion similar as described earlier for K41 (shown for P1/33/2 in Fig. 2 B).

Ultrastructural analyses by SEM revealed that the cellular membranes between contacting cells in the absence of mAb K41 are predominantly smooth and express only few microvilli-like structures (Fig. 5 A). Also in the presence of mAb H110 to CD9 the cell-cell contact areas remained unchanged (Fig. C). In contrast, after 2, 6, and 20 h incubation with mAb K41, numerous microvilli-like membrane protrusions appeared at the cell contact areas (Fig. 5 B, D, E). These microvilli along the cell-cell contact areas increased in length and became smaller in diameter between 2 and 20 h post induction. Ultimately they ended up being approximately 0.2-0.4 μm thick and several μm long. Thus, CD9 clustering supported the formation of microvilli-like membrane protrusions at cell-cell contact areas, which were observed already 2 h after incubation of cells with mAb K41, and which mature within the next 20 h to form zipper- or velcro-like structures between contacting cells.

Displacement or inclusion of viral proteins in CD9 clusters

Certain cellular transmembrane proteins such as EWI-F, which links TEMs to the actin cytoskeleton, directly interact with CD9 (6,9,29), whereas CDV envelope proteins do not. In spite of this lack of direct interaction, mAb K41 strongly inhibits the CDV-induced cell fusion (20,21). We hypothesized that the K41-induced clustering of CD9 may alter the composition of the plasma membrane and TEMs at cell contact sites, and this may restrict the capacity of the cell to undergo membrane fusion. To assess this, we analyzed the localization of viral proteins of CDV and MV (Fig. 6), and of the CD9-associated cellular molecules EWI-F, β 1-integrin (supplementary Fig. 2), and of cadherins (not shown) as controls, together with CD9 in cells before and after K41 treatment. EWI-F co-localized with CD9, independent of whether the tetraspanin is clustered or not (supplementary Fig. 2 C, F), whereas β 1-integrins and cadherins co-localized only partially, but were also present in large CD9 clusters (supplementary Fig. 2 I, L).

To investigate if linkage of tetraspanins and the cytoskeleton by EWI-F is a prerequisite for the formation of CD9 cluster and net-like structures, we reduced the EWI-F expression with EWI-F-specific siRNA. By two consecutive transfections of HeLa cells with siRNA we achieved a maximal reduction of the EWI-F expression by 83%. When the cells were treated under these conditions with mAb K41, we observed the unchanged formation of CD9 net-like structures after 2 (supplementary Fig. 3) and 20 h suggesting that EWI-F interaction is not required for the formation of this tetraspanin structure.

CDV- and MV-infection of Vero cells in the absence of K41 induced syncytium formation beginning approximately 12 h post infection. At 24 h post infection viral proteins were found predominantly in large perinuclear clusters, but also in minor amounts at cell membranes. CDV and MV proteins partially co-localized with CD9 in the cytoplasm (Fig. 6 A-C and G-I, respectively). When infected cells were simultaneously treated with K41, CD9 clustering and the above described characteristic net-like structures at cell contact sites were induced. Interestingly, no CDV proteins were detected in the large net-like CD9 clusters, whereas they continued to co-localize with CD9 within the cell in areas distant from cell contact sites (Fig. 6 D-F). In contrast, in MV-infected cells the cell-cell fusion is not inhibited by K41. In these cells viral proteins were found also within large CD9 clusters (Fig. 6 J-L). Similar results were obtained using cells transfected with expression vectors for the envelope proteins H and F (fusion protein) of CDV and MV (not shown). Thus, K41-induced CD9 clusters displace CDV envelope proteins, but not MV proteins, from cell-cell contact areas, and by excluding these viral proteins CD9 clusters prevent interactions

between the viral fusion machinery and the target cell membrane, which are necessary for virus-induced cell-cell fusion and cell-to-cell spread of virus.

DISCUSSION

The monoclonal antibody K41 induces a rapid relocation and clustering of CD9 at cell contact sites, which is associated with the formation of microvilli-like protrusions between contacting cells. Co-localization of the other tetraspanin family members CD63, CD81, and CD82 and proteins like EWI-F with CD9 in K41-induced clusters suggests that this mAb can alter the overall organization of surface TEMs. In infected cells, this may lead either to a physical separation of the viral fusion machinery from cell-cell contact areas, or to the inclusion of viral envelope proteins, depending on the virus investigated. Cell-cell fusion may result in giant cell formation, as observed *in vivo* during measles, or may be restricted to a transient micro fusion pore. Since viruses within a host often exploit this contact dependent way for cell-to-cell spread, CD9 may play an important role in viral pathogenesis.

After antibody interaction, the relocation and clustering of CD9 is a relatively fast event occurring within 1-2 h, whereas the formation of microvilli-like protrusions begins with the appearance of the characteristic large net-like CD9-positive structures after 2-3 h and proceeds over a longer period up to 20 h. The mechanism of protrusion formation at CD9-enriched membranes remains unclear. CD9-K41-complexes may bend the membrane into globular structures as detected in the laser scanning microscope and SEM, and together with the underlying actin cytoskeleton this may provide platforms for the formation of microvilli. Interestingly, the protrusions are observed exclusively at cell-cell contact areas, and not on contact-free surface membranes. They resemble those actin based “zippers” or “Velcro”-like structures described for epithelial cell layer repair during development and wound repair (for review see (30)). Our finding that upon treatment with latrunculin B the actin cytoskeleton is disrupted, microvilli zippers collapse and the cells retract from contacts, underlines that the cytoskeleton plays an important role in formation of these microvilli zippers. In the absence of a functional cytoskeleton the CD9 net-like structures collapsed. However, we could not detect actin fibers within the protrusions induced by K41, and it is not known whether they are involved in cell adhesion.

Beyond the tetraspanins, CD9 has predominantly been associated with cell motility and membrane fusion. CD9 enables the gamete-sperm fusion and CD9-deficiency results in reduced female fertility (12-14,31). However, CD9 may also inhibit cell fusion under certain circumstances as demonstrated for mononuclear phagocytes (32). What is the role of CD9 in virus induced cell fusions and cell-to-cell spread of viruses? Interestingly, adverse effects of antibodies to CD9 have been described for the cell-cell fusion induced by CDV, MV and HIV-1. While certain CD81 and CD9 antibodies enhance HIV-induced cell fusion (19), other antibodies such as K41 inhibit HIV budding and release of virus particles. Although tetraspanins have been used primarily as markers for late endosomal structures and multivesicular bodies, recent studies indicated that in T cells as well as in macrophages tetraspanins are taking part in the assembly and budding of HIV at the plasma membrane and other cellular, seemingly intracellular membranes, which may reflect a function in the viral morphogenesis pathway (17,33,34).

Our results provide an explanation for the specific effects on the CDV-induced and lack of effect on the MV-induced cell fusion, and suggest corresponding consequences for other viruses. The data indicate that the structural alterations of the membrane alone do not inhibit the virus-induced cell-cell fusion, but rather the displacement of virus receptors is decisive. We have shown earlier using chimeric CDV/MV envelope proteins that the viral haemagglutinins are responsible for these specific effects (28). Since the viral

haemagglutinins do not directly interact with CD9 (20), it is likely that the cellular receptors (in this case CD46 for MV, and unknown for CDV) play a decisive role in mediating the observed effects. It should be mentioned that virus-cell fusion and virus entry are not affected by K41 (21). Furthermore, the possible role of CD9 in virus infections *in vivo* remains obscure. Interestingly, the capacity of CDV to form syncytia (cell-cell fusion) in cell culture correlates with attenuation *in vivo* (35,36), which probably reflects the usage of cellular receptors and target cell tropism.

The induction of CD9 clustering by antibodies is epitope dependent, since only certain antibodies such as K41, MM25/7, ALB-6, and P1/33/2 are functional, while other CD9 antibodies, even upon crosslinking with secondary antibodies, do not induce similar effects. MAb K41 recognizes a reducing agent-sensitive epitope on the LEL of CD9, and induces its effect in the absence of crosslinking secondary antibodies, whereas monovalent Fab fragments of K41, also after addition of crosslinking secondary antibodies, cannot fulfill this task. This suggests that the three dimensional structure of the bivalent antibody provides a certain distance or angle of interaction which is critical. Functional domains and epitopes of CD9 have already been defined (3,37,38). The LEL of CD9 bears a cystein-cystein-glycine motif, which presumably interacts with associated transmembrane receptors such as $\alpha\beta 1$ -integrin, and is influenced by the activation state of the associated integrin. According to our data it may be possible that bivalent mAb K41 is active by linking two CD9 molecules together to form homodimers, and in addition this interaction may force the LELs of CD9 to stay in a certain conformation. However, since CD9 homodimers are already assembled from newly synthesized proteins in the Golgi, appear at the cell surface, and may serve as building blocks for the assembly of larger multicomponent tetraspanin protein complexes (39,40), it is more likely that mAb K41 crosslinks preexisting CD9 dimers to form tetramers or multimers. Further investigations are required to characterize the structural and functional consequences of K41 binding, and might reveal mechanistic details of the subsequent CD9 aggregation and formation of microvilli zippers at cell-cell contact areas.

The causative relationship between CD9 aggregation and the formation of microvilli-like protrusions remains unresolved. CD9- and CD81-associated transmembrane receptors such as EWI-proteins, members of a novel subfamily of the Ig-superfamily (6,9,41), may provide mechanistic suggestions. They contain a stretch of basic charged amino acids in their cytoplasmic domains that interacts with ezrin-radixin-moesin (ERM) proteins, and acts as linker to connect TEMs with the actin cytoskeleton (29). We observed that EWI-F expressed on the cell surface co-clusters with CD9 at cell-cell contact areas in response to K41 treatment, and may there provide the link to the actin cytoskeleton. However, our finding (supplementary Fig. 3) that EWI-F knock-down does not interfere with the formation of tetraspanin clusters support the idea that formation of these clusters is mainly driven by the antibody interaction on the cell surface. The high antibody concentration necessary to induce CD9 clustering (5-15 $\mu\text{g/ml}$) supports this suggestion. Further investigations are required to elucidate the function of actin, ERM proteins, and signalling for microvilli formation at the sites of CD9 clustering. Interestingly, CD9 is also enriched on microvillar membranes of oocytes and regulates their shape, distribution, and clustering with other tetraspanins and still unknown surface proteins involved in human and mouse gamete fusion (42,43). Our findings underscore the relevance of CD9 for healthy and pathogenic cell-cell fusion processes, and may open interesting strategies to influence the cell-to-cell spread or release of specific viruses.

MATERIALS AND METHODS

Cell culture and virus

Vero cells (African green monkey kidney cells, ATCC CRL6318), HeLa cells (ATCC CCL2), and CD9-negative chinese hamster ovary (CHO-K1) cells were cultured in minimal essential medium (MEM) containing 5 % fetal calf serum (FCS), penicillin and streptomycin. Primary human umbilical vein endothelial cells (HUVEC) were prepared from umbilical cords obtained from the maternity ward of the University Hospital, Würzburg, as described (44). HUVEC were cultivated in M199 medium (Gibco) containing 25 mM HEPES, 20% FCS (Biobrom), 5 U/ml Heparin, 30 µg/ml endothelial cell growth supplement (ECGS, Sigma), and 100 U/ml penicillin/streptomycin (45). CDV strain Onderstepoort large plaque and MV strain Edmonston were propagated using Vero cells (20).

Antibodies and immunostaining

Mouse monoclonal antibodies clone K41 (IgG1k) against CD9 (20), and clone 38/87 to moesin (a kind gift of R. Schwartz-Albiez, Deutsches Krebsforschungszentrum, Heidelberg, Germany), were produced in our laboratory. Hybridoma cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. MAbs were purified by protein-G affinity chromatography according to standard procedures. Fab fragments were prepared using the Immunopure Fab Preparation Kit (Pierce), and their purity was controlled by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We used fluorescein isothiocyanate (FITC)-conjugated CD9 mAb clone P1/33/2 (DAKO), clone MM2/57 and ALB-6 (Abcam), clone H110 (Santa Cruz), mouse anti-human CD29 (β1-integrin)-Alexa-647 (Serotec), Alexa-594-conjugated F(ab')₂ fragment of goat anti-mouse IgG (Molecular Probes Invitrogen), and Fcγ-specific Fc-CyTM2-conjugated affinity pure goat anti-mouse IgG (Dianova). Mouse mAbs to EWI-F were a kind gift of E. Rubinstein, INSERM, Hopital Paul Brousse, Villejuif, France (6). Mouse mAbs to CD63 (H5C6), CD81 (Z81.1) were a kind gift of F. Lanza, Etablissement de Transfusion Sanguine de Strasbourg, France, and anti-CD82 was from NatuTec. Polyclonal dog anti-CDV hyperimmune serum was a kind gift from M. Appel, Cornell University, Ithaca NY, USA, and was used in a 1:1500 dilution. MV specific hyperimmune serum from a patient with subacute sclerosing panencephalitis was used in a 1:6000 dilution. Goat anti-human FITC and goat anti-dog FITC were obtained from Sigma-Aldrich, goat anti-mouse FITC and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies from DAKO and Immunotech, and goat anti-mouse Alexa-594 and goat anti-rabbit Alexa-488-conjugated antibodies, and Alexa-488-conjugated phalloidin from Molecular Probes. To visualize CD9 aggregates, cells were fixed with paraformaldehyde for 20 min at room temperature, and then permeabilized with 0.1% Triton X100 for 10 min on ice.

Inhibitors

Inhibitors were: latrunculin (up to 100 mM, Calbiochem), against the small GTPases (up to 200 µM) Rho (Y-27632, Sigma), Rac (Rac1-inhibitor, Calbiochem), CDC42 (up to 1 µg/ml lethal toxin of *Clostridium sordellii*, toxin B of *Clostridium difficile*, kindly provided by Dr. K. Aktories, University of Freiburg, Germany), a PI3-kinase inhibitor (up to 1 mM), a PI4-kinase inhibitor (phenyl oxide, up to 100 nM), a PKA inhibitor (adenosine-3',5'-cyclic monophosphothioate, up to 200 µM), and a PKC inhibitor (Ro-32-0432, up to 550 nM), all from Calbiochem.

Short interfering RNA

Control siRNA against GAPDH, non-functional control siRNA #1 (siRNA Starter Kit containing siPORT NeoFX transfection agent, Ambion), and three predesigned siRNAs against EWI-F (target gene symbol: PTGFRN; siRNA-1 ID # 147749 5'->3' GCCCGUUUUUAUAACUGUGtt, siRNA-2 ID # 147750 5'->3' CCUCAGGUCCUAUAUUUAAtt, siRNA-3 ID # 147748 5'->3' GGCCACUACAAAUGUUCAAtt) were purchased from Ambion. 5×10^4 HeLa cells were seeded on 4 chamber slides and after 24 h transfected with 30-100 pmol siRNA. To achieve maximal reduction of EWI-F, the transfection was repeated after 2 days. The expression of EWI-F was analyzed by flow cytometry. The transfection efficiency was controlled by using directly FITC-labelled siRNA (Ambion).

Expression plasmids

Expression plasmids for CD9 and chimera with CD81 and CD82 were a kind gift of Eric Rubinstein, INSERM, Hopital Paul Brousse, Villejuif, France, are described in (38). The expression plasmid of CD9-eGFP, cloned in the vector eGFPN1 (Clontech) was a kind gift of Martin Hemler, Dana-Farber Cancer Institute, Boston, USA. HeLa cells stably expressing CD9-eGFP were selected using 800 $\mu\text{g/ml}$ G418. Clones expressing the protein were isolated using a cloning cylinder and were maintained in medium containing G418. Expression plasmids of CDV (strain Onderstepoort) and MV (strain Edmonston) H and F proteins in pCG vector are described in (28).

Flow cytometry and confocal microscopy

Flow cytometric analyses were performed on a FACS-calibur (BD) using standard procedures. For confocal laser scanning microscopy we used the Laser Scan Microscope, LSM510 Meta (Zeiss) with software version 3.2 SP2 (Zeiss), and an Axiovert 200 microscope, objective x40, aperture 1.4 plan apochromat. When indicated, vertical z-stacks were acquired (20 optical planes, 0.5 μm distance), and 3D deconvolutions and 3D reconstructions were performed by using the Zeiss software. For timelapse analysis HeLa cells stably expressing CD9-eGFP were plated in 2-chambered coverslips (Labtek) and imaged 2 minutes after addition of fresh medium containing 15 $\mu\text{g/ml}$ of K41. A series of three 2 μm optical slices was captured every 2 min for the first 2 h, then every 5 min for the next 4 h. Only bottom sections are shown in the timelapse analyses. Cells were imaged on a DeltaVision Workstation (DV base 3/3.5, Nikon Eclipse TE200 epifluorescence microscope fitted with an automated stage, Applied Precision Inc., Issaquah, WA, USA). Cells were maintained at 37°C and 5% CO_2 using a stage-enclosing Weatherstation climate chamber (Applied Precision Inc). A 60X Nikon Plan Apo 1.40 NA objective was used. Images were post-processed to digitally reassign out of focus light using Softworx deconvolution software (Applied Precision Inc.).

Scanning electron microscopy (SEM)

For SEM, cells were cultured on glass coverslips, fixed with 6.25% glutaraldehyde in 50 mM phosphate buffer (pH 7.2) for 10 min at room temperature and subsequently at 4°C overnight. After a washing step in PBS, samples were dehydrated stepwise in acetone, critical point dried, and sputtered with platinum/palladium before SEM analysis (Zeiss DSM 962).

Western blotting

Cells (1×10^6) were lysed in Lysis buffer (1 % NP40, 0.125 M NaCl, 1 mM EDTA, 50 mM HEPES) containing protease inhibitors (Boehringer) and applied in non-reducing protein sample buffer to 14% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were

blotted semi-dry on nitrocellulose. Nitrocellulose sheets were blocked with 5% dry milk in phosphate buffered saline (PBS) containing 0.05% Tween20 and incubated with 1 µg/ml mAb K41 and HRP-conjugated secondary antibodies (1:2000; Immunotech). Signals were visualized using the ECL system (Amersham).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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LIST OF ABBREVIATIONS

CDV	canine distemper virus
HIV	human immunodeficiency virus
H	haemagglutinin
LEL	large extracellular loop
MV	measles virus
TEM	tetraspanin enriched microdomain

Figure 1. (A-J) Distribution of CD9 on the cell surface before and after incubation with CD9 antibody K41

Vero, HeLa and primary human umbilical vein endothelial cells (HUVEC) were incubated at 37 °C in the absence (A, C, E) or presence (B, D, F) of 15 µg/ml mAb K41 for 20 h. Cells were fixed, permeabilized, and stained with secondary antibodies conjugated to Alexa-594 (red). Arrow heads in B, D, F designate the large CD9-enriched clusters at cell-cell contact areas appearing as net-like structures in Vero and HUVEC, and as more compact aggregates in HeLa cells. Vero cells were incubated in the absence (G, I) or presence (H, J) of anti-CD63 (G, H) and CD81 antibodies (I, J; 15 µg/ml) for 20 h, and then stained with the same antibodies to show the distribution of these molecules as controls.

(K) Recognition of the large extracellular domain (LEL) of CD9 by mAb K41. Vero cells were transfected with expression plasmids for CD9, CD81, CD82 and chimera as indicated, and the empty vector as negative control, stained with K41 and FITC-conjugated secondary antibodies, and the mean fluorescence intensities determined by flow cytometry. The mean fluorescence intensity of the mock-transfected CHO cells was normalized to 1 and the relative fluorescence intensities of the assessed samples were presented in relation to the control ($\pm$ SD; n = 3).

Figure 2. Functional analysis of K41 Fab fragments

(A) Flow cytometry of Vero, HeLa and HUVEC stained with complete K41 (black line; 5 µg/ml), K41 Fab-fragments (dashed line; 2 µg/ml), and isotype control (grey filled curve; 5 µg/ml), and FITC-conjugated secondary antibodies. (B) Quantification of the CDV-induced syncytium formation of Vero cells in the presence of K41 and Fab fragments. Vero cells were transfected with CDV H and F expression vectors, and after addition of antibodies incubated for 20 h at 37°C. The average number of cell nuclei in syncytia was determined in the microscope by counting 5 random fields in 3 independent experiments ($\pm$ SD; $n = 3$), and the data were normalized to the control (lane 1; no antibody) being 100%. Lane 2 and 3: 5 and 15 µg/ml K41; lane 4: 10 µg/ml clone P1/33/2 against CD9; lane 5: 5 µg/ml K41 Fab fragments; lanes 6 to 8: 5 µg/ml Fab fragments for 1 h plus 2, 5, and 10 µg/ml secondary crosslinking antibody; lane 9: 10 µg/ml secondary antibodies alone.

Figure 3. Kinetics of CD9 clustering and steady state levels of CD9

(A) HeLa cells transfected with CD9-GFP were treated with 10 µg/ml K41 at 37°C and the distribution of CD9 was documented after 0-6 h as indicated. Arrows indicate a large net-like CD9 cluster at a cell-cell contact area. (B) Lysates of Vero, HeLa, and HUVEC (lanes 1-4, 5-8, 9-12, respectively) were prepared 0, 2, 6, and 20 h after treatment with K41. After separation on a 14% SDS-polyacrylamid gel under non-reducing conditions and blotting to nitrocellulose, CD9 bands were visualized using mAb K41, HRP-conjugated secondary antibodies, and the ECL system. Moesin bands are shown as control of the amount of cell lysates loaded.

Figure 4. Globular CD9-enriched structures in contact areas between K41-treated cells

A typical net-like CD9-enriched cell-cell contact area of K41 treated Vero cells (A) is shown further enlarged in (B) and three dimensionally reconstructed in (C). Double staining of the cells for CD9 (goat anti-mouse Alexa-594, red) and actin (phalloidin Alexa-488, green) is shown in (D, E). Similar net-like CD9 clusters as in Vero cells were detected in HUVEC (F, G) and HeLa cells (H, I) after treatment of the cells with 15 µg/ml mAb K41, and also after treatment of cells with FITC-conjugated anti-CD9 mAb P1/33/2 (J, K), but not after treatment of cells with anti-CD9 mAb H110 (L, M).

Figure 5. Net-like CD9 clusters consist of microvilli-like protrusions

Vero cells were incubated without antibody (A), with 15 $\mu\text{g}/\text{ml}$ mAb H110 (C), and 15 $\mu\text{g}/\text{ml}$ with mAb K41 (B, D, E) for 2 h (A, B), 6 h (C, D) and 20 h (E) and processed for analysis by scanning electron microscopy (SEM). In the right column, enlargements of the left column panels are shown as indicated. Arrows in A and B designate the location of the cell-cell borders of two contacting cells.

Figure 6. Displacement of CDV proteins, but not MV proteins, from K41-induced CD9 clusters
 Vero cells were infected for 24 h with CDV (A-F), or MV (G-L), in the absence (A-C; G-I) or presence (D-F; J-L) of K41. A CDV-induced syncytium containing three nuclei in the absence of K41 is shown in A. Viral proteins were stained using dog anti-CDV or human anti-MV hyperimmune sera and FITC-conjugated secondary antibodies (green), and CD9 using goat anti-mouse Alexa-594 (red). Enlargements of A, D, G, J (squares) are shown in B, E, H, K. Profiles of the fluorescence intensities of the red and green fluorescences along the arrows in A, D, G, J, (representing 35 μ m) are presented in C, F, I, L, respectively. In CDV-infected cells, viral proteins are not detected in the large net-like CD9 clusters, but co-

localize within the cell (F). In MV-infected cells, viral proteins partially co-localize with CD9 irrespective of whether the cells were treated with K41 or not (I, L).