Structural and Functional Analysis of Integrin α₂I Domain Interaction with Echovirus 1*

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Integrins are cell surface receptors for several microbial pathogens including echovirus 1 (EV1), a picornavirus. Cryo-electron microscopy revealed that the functional domain (α₂I) of human α₂β₁ integrin binds to a surface depression on the virus capsid. This three-dimensional structure of EV1 bound to α₂I domain provides the first structural details of an integrin interacting with a picornavirus. The model indicates that α₂β₁ integrin cannot simultaneously bind both EV1 and the physiological ligand collagen. Compared with collagen binding to the α₂I domain, the virus binds with a 10-fold higher affinity but in vitro uncoating of EV1 was not observed as a result of attachment of α₂I. A molecular model, constructed on the basis of the EV1-integrin complex, shows that multiple α₂β₁ heterodimers can bind to adjacent sites around the virus 5-fold symmetry axes without steric hindrance. In agreement with this, virus attachment to α₂β₁ integrin on the cell surface was found to result in integrin clustering, which can give rise to signaling and facilitate the initiation of the viral entry process that takes place via caveolae-mediated endocytosis.

Numerous clinically important viruses, including adenoviruses, hantaviruses, and picornaviruses, use the members of the integrin family as their cellular receptors. The natural ligands of the integrins include many extracellular matrix proteins, and the physiological function of these receptors is to mediate cell adhesion. Integrins are heterodimers, consisting of an α-subunit and a β-subunit. Following ligand binding, they generate cellular signals leading to the formation of cytoskeletal connections and specific cellular responses (1). It is likely that the general properties of integrins have facilitated their utilization as receptors for a number of viruses. However, the mechanisms by which viruses have evolved to utilize cell surface integrins for binding and internalization remain largely unknown because insufficient structural information has been available on the details of virus-integrin interactions.

Echovirus 1 (EV1), a member of the Picornaviridae family, uses the α₂β₁ integrin (VLA-2) as its receptor for cell entry (2) through caveolae-mediated endocytosis (3). In humans, echovirus infections are associated with meningitis, encephalitis, rash, respiratory infections, diarrhea, and even fatal illness in newborns (4). The picornavirus particle is composed of a single-stranded, infectious RNA molecule, which is packed into an icosahedral capsid consisting of 60 proteomes, each containing four viral proteins (VP1–4) (5). A surface depression termed the canyon is located around each of the twelve 5-fold axes of the capsid in many picornaviruses. The canyon has been shown to be a binding site for receptor molecules that belong to the immunoglobulin superfamily (6).

The binding site of EV1 on α₂β₁ integrin has been located to the inserted domain of the α-subunit (α₂I) (7), which also forms the binding site of the physiological ligand collagen. The residues identified as essential for EV1 binding are mostly positioned on a face of α₂I different from the collagen binding site (8, 7). The binding mechanisms of these two ligands to α₂β₁ integrin are evidently different: in contrast to collagen binding to the integrin, the attachment of EV1 has been reported to be independent of divalent cations (8).

Here, we have determined the structure of the complex of EV1 and α₂I domain by cryo-electron microscopy (cryo-EM) and compared the interactions in the receptor-virus complex with those made to collagen (10). The interaction with α₂I domain does not appear to directly induce uncoating of the virus, as is the result of poliovirus receptor interactions. Moreover, following the binding of EV1, we have observed clustering of α₂β₁ integrin on the plasma membrane, which is essential for the activation of integrin-related signaling pathways (11). These mechanisms may have key implications for initiation of infection by viruses that interact with cell surface integrins.

EXPERIMENTAL PROCEDURES

Preparation of Viruses and Receptors—Human α₂ integrin I domain was produced as a glutathione S-transferase-α₂I (GST-α₂I) fusion protein as described earlier by Ivaska et al. (12). A soluble construct of two poliovirus receptor (PVR) molecules linked together by an Fc antibody fragment (PVR-Fc) was prepared as described by Xing et al. (13). EV1 (Farouk strain) and poliovirus 1 (PV1, Sabin strain) were obtained from the American Type Culture Collection (ATCC) and propagated in African green monkey kidney (GMK) (ATCC) cells. To obtain radioactively labeled preparations of EV1 and PV1, infected GMK cells were incubated in the presence of [35S]methionine (50 μCi/ml; Amersham Biosciences) in Eagle’s minimal essential medium (MEM) deficient in t-methionine (Invitrogen Life Technologies, Inc.). The viruses were purified by sucrose gradient sedimentation as described previously (14).

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1 The abbreviations used are: EV, echovirus 1; GST, glutathione S-transferase.
and suspended in phosphate-buffered saline containing 2 mM MgCl2.

Purified virus preparations were examined by neutralization using

serotype-specific antisera, and the infectivity of the viruses was deter-

mined by a plaque titration assay. Protein concentration of the EV1

preparations was measured using BCA Protein Assay Kit (Pierce Bio-

technology), and the purity of the capsid proteins was analyzed on a

12% SDS-PAGE gel.

Molecular Modeling—The crystal structure of unliganded αI domain was

fit manually into a difference density map, computed by subtract-

ing the crystal structure-based density of native EV1 (Protein Data

Bank (PDB) entry 1EV1) (5) from the αI-domain complex map, using

the interactive mode of the software BODIL (www.abo.fi/fak/mnf/bkf/bodil.html). An exhaustive search of the local trans-

lational and rotational space was then performed in BODIL. The sam-

pling parameters were as follows: 10° rotational step around 321 axes

spaced 7.9° from one another, giving an even sampling of the solu-

tion space. These parameters resulted in 3.96 million transformations.

The value of f was based on the sum of map density values at indi-

vidual Cα atom positions for each transformation of αI domain in the

search. Positions with Cα atoms overlapping the high-resolution crystal

structure of EV1 were discarded, and the remaining solutions were

scored according to their fit to the cryo-EM density. The correspondence of the N terminus of αI domain with the linker density to the GST

protein was used as a filter to register the search results. The ten

highest scoring solutions were essentially identical to the manual fit (all

pairwise root mean-squared deviations, computed for the Cα atoms,

were less than 2.3 Å), which was chosen as the model described here.

A comparative model of the αβ2 integrin heterodimer was built

using the crystal structure of the αβ2 ectodomain (PDB entry IJ2V)

with the aid of bound anti-α2 integrin (MCA2025 from Serotec Inc.) and then with a cross-linking Alexa488-conjugated

anti-mouse IgG antibody (Molecular Probes Inc.), each for 1 h on ice.

This reconstruction was further refined with the data from the close-

end region of the integrin (1): three N-terminal domains of the

α-subunit (β-propeller domain, I domain, and Ig-like domain) and two N-terminal domains of the

β-subunit (β domain and Ig-like domain).

Conformal Immunoassay Microscopy—In vivo crosslinking of the

αI domain and Ig-like domain).

αI domain (13, 20, 21). Incubation of PV1 alone

slightly (Fig. 1B) to PV1 dramatically altered the conformation of the poliovirus

capsid and induced the release of viral RNA, resulting in 80 S

empty particles (Fig. 1B). The presence of PV1 with GST or

GST-αI caused no inhibition of infection (data not shown).

A sucrose gradient sedimentation assay was used to identify possible conformational alterations of the viral capsid structure after

interaction with the receptor. At 4 °C, the EV1 capsid remained intact and sedimented at 160 S, whereas at 37 °C, a significant portion of EV1 spontaneously formed empty parti-

cles, which sedimented at 80 S (Fig. 1B). The presence of GST-αI fusion protein altered the sedimentation of EV1 slightly (Fig. 1B), but the virus did not form 80 S particles

(which lack both VP4 and viral RNA); these sedimentation coefficients correspond to the subviral particles obtained from

PVR-bound poliovirus (13, 20, 21). Incubation of PV1 alone

either at 4 °C or at 37 °C for 15 min yielded only intact 160S particles (data not shown), whereas the attachment of PVR-Fc to

PV1 dramatically altered the conformation of the poliovirus

capsid and induced the release of viral RNA, resulting in 80 S

particles (Fig. 1B). Thus, the in vitro binding of a soluble receptor fragment to EV1 does not induce significant confor-
mational changes in the viral capsid but rather inhibits forma-
tion of 80 S empty particles.
**α₂I Domain Binds within the Virus Canyon**—We have determined the structure of the α₂I domain bound to EV1 by cryo-EM. The complex was prepared by mixing virus with the GST-α₂I fusion protein for 3 h at 4 °C. The fusion protein tends to form intermolecular disulfide bonds, which resulted in aggregates that were observed in cryo-electron micrographs (data not shown). Thus, β-mercaptoethanol was added to the buffer to a final concentration of 2 mM during incubation in order to maintain the fusion protein in a monomeric form. Consequently, virus-receptor complexes appeared in micrographs as individual spherical particles and the three-dimensional structure of the complex was reconstructed based on icosahedral symmetry.

The reconstruction of the virus-receptor complex revealed an EV1 particle decorated with 60 copies of the α₂I domain (Fig. 2B). The bound receptor is located roughly halfway between the viral 2-fold and 5-fold axes. The receptor density extends to a radius of 180 Å, possesses a density value similar to that of the viral proteins, and has a volume that is sufficient to accommodate only the α₂I domain of the fusion protein. A peptide linker of 20 residues connects the GST domain to the N terminus of the α₂I domain in the fusion protein. The lack of discernible GST density is likely due to the flexibility of this hinge region. When the density map was examined at low isodensity contours (0.3–0.5 σ), the linker could be traced to a tubular density extending from the α₂I domain toward the viral 3-fold axis (not shown). A difference map was generated by subtracting the crystal structure based density for native EV1 (Fig. 2A) from the cryo-EM density map of the virus-receptor complex (Fig. 2B). The unliganded α₂I structure (PDB entry 1AOX) (22) was docked into this density-difference map. The crystal structure of the α₂I domain in complex with a synthetic collagen-like triple helical peptide (PDB entry 1DZI) (10) has also been solved, showing a large conformational change at the collagen binding site. Since collagen was not present in our experimental conditions, the unliganded α₂I domain crystal structure was presumed more representative and was chosen for fitting to the difference density.

In the resulting model, the α₂I domain binds within the canyon on the EV1 surface with extensive contacts to the outer canyon wall (Fig. 2C), but the domain is not in intimate contact with the inner canyon wall. In the docked position, both the N and C termini are exposed to the solvent and point away from the virus surface. This agrees with the situation in αβ3 integrin where both termini of the α₂I domain are connected with the seven-bladed β-propeller structure in the α-subunit (16). The α3 helix of the α₂I domain together with the connecting loops interact with the capsid protein VP2 from one protomer (on the
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FIG. 2. Interaction between α₂I domain and EV1. A, the EV1 density map was generated from the crystal structure of the native virus. The icosahedral symmetry axes are labeled with corresponding numbers. B, a density contour presentation of the three-dimensional reconstruction of α₂I-bound EV1. C, structure of α₂I domain (blue ribbon) fit into the difference density map (transparent gray). α₂I domain interacts with two virus protomers (gold and light yellow surfaces). One key residue, Tyr216, is located above the protomer interface (red ball-and-stick model). The icosahedral 5-fold and 3-fold axes and the N’ and C’ termini of α₂I domain are labeled. D, electrostatic charge distribution on the virus and α₂I domain surfaces (negative, red; positive, blue; neutral, white). α₂I domain (top) is removed from the docked position and rotated by 180°. Charge-complementary residues are labeled and connected with lines. Bar, 100 Å.

left in Fig. 2C), while the end opposite to the N and C termini contacts VP3 from a neighboring protomer (on the right in Fig. 2C). The metal ion-dependent adhesion site (MIDAS) points toward the canyon floor but it is not in close contact with the virus.

The Virus-Receptor Interface—There are in total three charge residues from the α₂I domain (Lys201, Asp219, and Arg268), lining the binding interface that could form complementary electrostatic interactions with EV1 (respectively with Glu2162-Asp2163, Lys2230, and Glu1273, viral residues are numbered sequentially starting from 1001, 2001, 3001, 4001 for VP1, VP2, VP3, and VP4, respectively). Studies of EV1 binding to chimeric and mutated α₂I domains have suggested that residues 199–201, 212–216, and 289 are required for EV1 binding (23, 24). Our fitting places residues 199–201 (Thr-Tyr-Lys) of the α₂I domain on the EV1 surface in the E-F loop located between the E and F strands of VP2, where Lys201 can form a favorable electrostatic contact with two negatively charged residues of the virus: Glu2162 and Asp2163 (Fig. 2D). The side chain of Tyr200 of α₂I is positioned to stack with the side chain of His2164 from EV1. α₂I domain interacts with two adjacent viral protomers, placing residues 212–216 (QTSQY) above the interface with the phenolic ring of Tyr216 pointing toward the viral surface (Fig. 2C). Arg268 and Asn269 in the αC-a6 loop of α₂I may interact with the C terminus of VP1 from the second protomer, where the positively charged side chain of Arg268 points toward the negatively charged Glu1273 side chain of the virus (Fig. 2D). The point mutation N289G is known to relax the binding specificity of α₂I toward different collagen types (24), even though this residue is not in close contact with the collagen triple helix (10). This mutation may result in the alteration of the dynamics of the βE-a6 loop of the α₂I domain, a region involved in a large conformational shift upon collagen binding, and it is likely that mutation of N289 influences virus binding through a conformational effect.

Collagen and EV1 Cannot Bind to the α₂I Domain Simultaneously—In our model of the EV1-α₂I complex, the MIDAS motif of α₂I domain, where collagen binds (10), points toward the canyon without direct contact with the viral surface. Superposition of the crystal structure of the α₂I domain in complex with the collagen-like peptide (Fig. 2A) (10) on the EV1-α₂I complex (Fig. 3B) shows that the collagen triple helix would occupy space overlapping that occupied by EV1 when bound to the α₂I domain. Thus, the model strongly suggests that the α₂I domain cannot bind both collagen and EV1 simultaneously and that, therefore, EV1 must compete with native ligands for free αβ₃ molecules. From the solid phase binding assay, the apparent Kd for the α₂I domain bound to EV1 is 2 nM (Fig. 3C), an affinity 10 times greater than that of the α₂I domain bound to collagen type I (≈20 nM). Again, the model structure for the EV1-α₂I complex and the crystal structure of α₂I in complex with the collagen-like triple-helical peptide are consistent with these experimental results: the solvent-accessible surface area of α₂I buried when bound to EV1 (850 Å²) is substantially larger than that buried by the collagen-like peptide (359 Å²).

EV1 Binding Activates Integrin Clustering—EV1 attachment to the αβ₃ integrin is known to initiate the subsequent internalization of both the virus and the integrin in caveolae (3), suggesting that concomitant integrin signaling may take place. The key question concerning the activation of signaling is whether the ligand can cause the clustering of integrins on the cell surface. In previous studies, integrin clustering has been induced with antibodies, which triggers the activation of signaling pathways and leads to the accumulation of cytoskeletal proteins at the sites of the clusters (11). We investigated the distribution of αβ₃ integrin on the cell surface by confocal immunofluorescence microscopy after incubating cells with antibodies and with EV1. Formation of αβ₃ clusters was observed after treating αβ₃-SAOS cells with an anti-α₂I antibody and a secondary cross-linking antibody (Fig. 4, A and B). Incubation of the cells with EV1 for 15 min caused a redistribution of αβ₃ similar to that induced by the antibody treatment (Fig. 4, C and D). Controls, where cells were incubated with either the anti-α₂I integrin antibody or an irrelevant anti-α₂I integrin antibody without cross-linking, did not result in changes in the distribution of αβ₃ (data not shown).

A comparative model for the αβ₃ integrin heterodimer (Fig. 5A) was constructed based on the crystal structures of αβ₃ and α₂I domain (16, 22) and then docked onto the EV1 structure by superimposing α₂I of the αβ₃ model on the α₂I domain of the virus-receptor complex structure. The five binding sites on the viral pentamer are able to accommodate the entire integrin molecule simultaneously without steric hindrance, despite the presence of the bulky 90 Å head of the αβ₃ heterodimer (Fig. 5B). Our three-dimensional model of the EV1-α₂I complex is compatible with the observed virus-induced integrin clustering: while the molecular model demonstrates that there is no steric obstruction to αβ₃ binding at adjacent protomers, binding of αβ₃ to sites separated by one or more protomers on the EV1 surface may also place the integrins in sufficient proximity to result in signal-inducing clusters.

DISCUSSION

The three-dimensional structure of EV1 bound to α₂I domain provides the first structural details of an integrin interacting with a picornavirus. The reconstruction shows that EV1 uses the canyon region for receptor binding, similarly to poliovirus and the major group of human rhinoviruses (HRVs), whose
receptors, PVR and intercellular adhesion molecule 1 (ICAM-1), belong to the immunoglobulin superfamily (IgSF) (13, 25, 26). In contrast, the low density lipoprotein receptor (LDLR), a receptor for the minor group of HRVs, and decay-accelerating factor (DAF), a receptor for echovirus 7, bind outside the canyon (27, 28). Despite binding into the canyon, the interactions of the ICAM-1 domain with the canyon surface of EV1 differ from those of the IgSF receptors. The ICAM-1 domain structure is larger and more globular than the elongated virus-binding domains of ICAM-1 and PVR. When we superposed the ICAM-1-EV1 complex onto the complex between ICAM-1 and rhinovirus 16 (29), the ICAM-1 domain footprint on the EV1 surface at the bottom of the canyon overlaps that of ICAM-1 on rhinovirus 16; however, the footprint of the ICAM-1 domain is larger and the ICAM-1 domain makes more extensive contacts with the outer canyon wall.

The effects of receptor binding on the conformation of EV1 are different from poliovirus and the major group of HRVs. It has been shown that binding of the soluble fragment of PVR to poliovirus and ICAM-1 to HRV is able to mediate conformational changes in the capsids of both viruses in vitro, resulting in the release of their genomes (21, 30). Our density gradient assay data suggest that in the case of EV1, attachment of the GST-ICAM-1 fusion protein is not sufficient to directly induce uncoating. Whether the action of αβ integrin on EV1 in vivo is identical to that of the GST-αI fusion protein and whether other cellular molecules are involved in EV1 uncoating requires further investigation.

EV1 binding to αIβ integrin represents the interaction of an αI domain containing integrin receptor with a picornavirus. Several other picornaviruses are known to use integrins as cellular receptors, but they recognize heterodimers that contain the αV subunit (no I domain) and interact with the arginine-glycine-aspartic acid (RGD) motif in the viral capsid protein VP1. These picornaviruses include coxsackievirus A9 and echovirus 9, which belong to enteroviruses, human parechoviruses and foot-and-mouth disease viruses (31–37). Interactions of adeno-viruses with integrins have been studied thoroughly. Adenoviruses attach to the cells first through a binding receptor (cox-sackievirus-adenovirus receptor or CD46) (38, 39) and subsequently recognize αV integrins with the RGD motif located in the viral penton protein (40). Binding of multiple
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Fig. 4. Attachment of EV1 to the cell supports the clustering of integrins. A, immunofluorescent labeling of α2β1 integrin (green) on α2β1-SAOS cells after 1 h of incubation with an anti-α2-integrin antibody. B, α2β1 integrin 15 min after antibody-mediated cross-linking. C, α2β1 on α2β1-SAOS cells after 15 min of incubation with EV1. D, co-localization of α2β1 integrin (green) and EV1 (red) is seen as yellow color. Bars, 10 μm.

Fig. 5. The EV1 pentamer can support clustering of integrins. A, a comparative model of the α2β1 integrin heterodimer with three domains of the α-chain (blue) and two domains of the β-chain (gold). B, five copies of the model (blue) were placed without steric hindrance around the virus (gold) 5-fold axis by superimposing α1 of the α2β1 model on the α1 domain of the virus-receptor complex structure.

Integrins to the virus particle causes clustering, which is known to induce integrin signaling that facilitates the internalization of the viral particle. Another example of virus-induced integrin signaling is the binding of Kaposi’s sarcoma-associated herpes virus (KSHV/HHV-8) to α2β1 integrin, which results in the activation of focal adhesion kinase (FAK) (41).

Crystal structures of two RGD-containing viruses have been determined, foot-and-mouth disease virus (FMDV) (PDB entry 1FOD) (42) and coxsackievirus A9 (CAV9) (PDB entry 1D4M) (14), as well as the structure of their receptor α2β1 integrin in complex with an RGD peptide (PDB entry 1L5G) (43). RGD-containing peptides bind at the interface of the β1 domain of the β-chain and the β-propeller domain of the α-chain, coordinating tightly to a metal ion of MIDAS in the β1 domain. In the FMDV capsid, the RGD sequence is located within the G-H loop of VP1, whereas in CAV9 it is near the C terminus of VP1 in a region that is not defined in the crystal structure of the virus. The details of receptor interactions with RGD-containing viruses must differ from those of EV1 with α2β1. Nonetheless, examination of the crystal structures of FMDV and RGD in complex with α2β1 by superposition of the RGD motifs, as well as modeling of the RGD-containing segment of CAV9 (not shown), suggests the possibility of integrin clustering since these two viruses can also accommodate multiple integrin heterodimers simultaneously at adjacent binding sites about the 5-fold axes.

EV1 accommodates α2β1 integrin in an orientation where the MIDAS motif faces the canyon floor, making it impossible for the receptor to bind collagen and virus simultaneously. The MIDAS surface is not in close contact with the virus structure, which is consistent with the finding that α2I domain can attach to EV1 in the absence of Mg2+ (9), in contrast to metal ion dependent interactions with collagen. The structure is also in agreement with the observation that a cyclic RKKH-containing octapeptide (12, 44), which binds to the MIDAS region (44) and blocks collagen binding to α2I domain (12), can bind the α2I domain simultaneously with EV1 and even increases the binding of α2I domain to the virus. There is sufficient space in our model at the MIDAS surface to accommodate the RKKH peptide. The enhancement of the binding probably reflects the shielding of the negatively charged residues around MIDAS by the peptide as well as the increase in the contact area between α2I domain and EV1 mediated by the peptide.

Since the model of the EV1-α2I complex precludes the simultaneous binding of collagen and EV1 to α2I domain, it implies that the virus must bind to cells that have ligand-free α2β1 integrin heterodimers on the cell surface or compete with native α2β1 ligands. Thus, the relatively low affinity of α2β1 integrin for its physiological ligands may be one of the factors that have driven receptor selection and allowed EV1 to successfully adapt to exploit this receptor for cell attachment. α2β1 integrin is usually expressed on the basolateral surfaces of cells but, interestingly, some cells such as skin keratinocytes may express α2β1 on surfaces that do not face basement membranes or any known α2β1 ligands (45). Both cellular entry routes may
play an important role in the pathogenesis of the clinical disease, since infection by EV1 is thought to take place primarily in the respiratory and gastrointestinal epithelium, but secondary virus replication occasionally is observed in various target organs.

In our model, all five binding sites around an EV1 5-fold axis can be occupied by integrin molecules without steric hindrance. This finding suggests that simultaneous binding of several αIβ1 integrins to the viral particle might also occur in vivo. Indeed, integrin clustering was observed during attachment of EV1 onto the cell surface, supporting the idea that this phenomenon leads to signaling events that may facilitate the entry of the virus. A similar phenomenon has been described in adenoviruses where multiple binding of integrins to the penton base pentamer induces clustering and gives rise to cellular signaling, having an important role in the internalization process (40). Likewise, EV1-induced integrin clustering can lead to the activation of signaling pathways and facilitate internalization of the virus via the caveolar pathway (46).

In conclusion, we report the structure of echovirus 1 in complex with the virus-binding domain of its receptor, αIβ1 integrin. To our knowledge, this is the first structural insight into picornavirus-integrin interactions. Interestingly, EV1 is the only picornavirus known to utilize αIβ1 integrin in cellular interactions and to use caveola-mediated endocytosis in the entry into the host cells (3), suggesting that the receptor interaction may largely determine the internalization route. The integrin molecule binds to the surface depression surrounding the 5-fold axes of EV1 in an analogous but not identical manner when compared with the interaction of receptors of the immunogoblin superfamily with polioviruses and rhinoviruses. The latter interactions give rise to in vitro uncoating, which is not seen after binding of αI to EV1. Whether the whole integrin molecule and additional receptors are needed for uncoating remains to be studied. It is possible that uncoating occurs later during caveolar entry, whereby the αIβ1 domain may stabilize the virus structure, insuring against premature genome release before EV1 has entered the targeted cell. The binding affinity of EV1 to αI is significantly higher than that of collagen, suggesting that the virus is able to efficiently compete for occupied basolateral binding sites. Both molecular modeling and in vitro experiments show that binding of an EV1 particle to multiple integrin molecules can cause integrin clustering, likely leading to cellular signaling, a central integrin function, as well as committing the virus-receptor complex to cell entry via the caveolar pathway.

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