Molecular mechanism of $\alpha 2\beta 1$ integrin interaction with human echovirus 1

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Conformational activation increases the affinity of integrins to their ligands. On ligand binding, further changes in integrin conformation elicit cellular signalling. Unlike any of the natural ligands of $\alpha 2\beta 1$ integrin, human echovirus 1 (EV1) seemed to bind more avidly a ‘closed’ than an activated ‘open’ form of the $\alpha 2$ domain. Furthermore, a mutation E336A in the $\alpha 2$ subunit, which inactivated $\alpha 2\beta 1$ as a collagen receptor, enhanced $\alpha 2\beta 1$ binding to EV1. Thus, EV1 seems to recognize an inactive integrin, and not even the virus binding could trigger the conformational activation of $\alpha 2\beta 1$. This was supported by the fact that the integrin clustering by EV1 did not activate the p38 MAP kinase pathway, a signalling pathway that was shown to be dependent on E336-related conformational changes in $\alpha 2\beta 1$. Furthermore, the mutation E336A did neither prevent EV1 induced and $\alpha 2\beta 1$ mediated protein kinase C activation nor EV1 internalization. Thus, in its entry strategy EV1 seems to rely on the activation of signalling pathways that are dependent on $\alpha 2\beta 1$ clustering, but do not require the conformational regulation of the receptor.

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Introduction

Adhesion receptors of the integrin family are known to anchor most cell types to the surrounding matrix. Several intracellular pathogens also bind to integrins to gain entry to the cell. Integrins are optimal virus receptors for several

reasons. They are abundantly expressed on the cell surface and they have relatively low affinity for their natural ligands. In addition, integrins are connected to signalling proteins that may trigger endocytotic pathways. Activation of integrin-mediated signalling is considered to be an essential mechanism for the internalization of viruses. In this process they may mimic the natural ligands.

The initial step in virus infection is binding of the virus particle to a specific receptor on the cell surface. Many adenoviruses (Wickham *et al*, 1993), coxsackievirus A9 (Chang *et al*, 1989; Roivainen *et al*, 1991, 1994; Williams *et al*, 2004), human parechovirus 1 (Hyypiä *et al*, 1992; Stanway *et al*, 1994; Joki-Korpela *et al*, 2001), foot-and-mouth disease virus (Fox *et al*, 1989; Jackson *et al*, 1997) and Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8; Akula *et al*, 2002; Veettil *et al*, 2008) have surface proteins harbouring an arginine–glycine–aspartic acid (RGD) motif, a well-known recognition sequence for a subset of integrins (Ruoslahti and Pierschbacher, 1987). Integrin $\alpha 2\beta 1$, a collagen receptor, binds to human echovirus 1 (EV1; Bergelson *et al*, 1992) and rotavirus (Zárate *et al*, 2000) in an RGD-independent manner.

Recent investigations have unveiled many essential facts concerning the structural basis of integrin signalling (for reviews, see Springer and Wang, 2004; Arnaout *et al*, 2005). Inactivated integrins are proposed to take a bent conformation. Activating ‘inside-out’ signals, such as talin or kindlin binding to β -integrin cytoplasmic domain, can trigger a conformational change leading to the extension of integrin ectodomain (for review, see Moser *et al*, 2009). Natural ligand binding to a site formed by the inserted domain of the β -subunit (β I domain) and the β -propeller domain of the α -subunit triggers a conformational change in the β I domain, leading to the separation of the α - and β -leg regions (Xiao *et al*, 2004). This results further in the dissociation of α - and β -cytoplasmic domains, allowing activation of intracellular signalling protein binding to the integrins. Multivalent ligands can, in addition to conformational changes, induce integrin cluster formation. Integrin-binding viruses have been thought to act in a manner similar to natural multivalent ligands. However, the results reported here indicate that EV1 diverges from all previously studied $\alpha 2\beta 1$ integrin ligands.

Integrin $\alpha 2\beta 1$, as well as the three other human collagen receptor integrins ($\alpha 1\beta 1$, $\alpha 10\beta 1$ and $\alpha 11\beta 1$) and the five leukocyte integrins ($\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha X\beta 2$, $\alpha D\beta 2$ and $\alpha E\beta 7$), belong to a structurally distinct subgroup of integrins. These nine α -subunits have a ligand-binding α I domain, homologous to the β I domain found in all the integrin β -subunits. The ‘I’ domains or ‘inserted’ domains are also called ‘A’ domains on the basis of their structural similarity to von Willebrand factor A domains (Arnaout *et al*, 2005). The αL and αM integrin α I domains can assume a closed, an intermediate or an open conformation (Shimaoka *et al*, 2003a), whereas in the $\alpha 1$ I and $\alpha 2$ I domains the intermediate

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form may not exist (Jin *et al*, 2004), suggesting that ligand binding to the latter domains triggers a change from the closed to the open conformation (Emsley *et al*, 2000). The open conformation is also detected in activated integrins before ligand binding, and it may represent a high avidity state of the αI domain. In a recombinant $\alpha 2I$ domain, the alteration from the closed to the open conformation can be induced by the gain-of-function mutation E318W (Aquilina *et al*, 2002). All natural ligands, including different collagen and laminin subtypes, have shown better binding to the open $\alpha 2I$ domain when compared with the closed domain form (Aquilina *et al*, 2002; Tulla *et al*, 2008).

The key mechanism involved in signalling by the $\alpha M\beta 2$ and $\alpha L\beta 2$ integrins seems to act through a glutamate residue, located close to the C-terminus of the $\alpha 7$ helix in the αI domain (E310 in αL and E320 in αM), which acts as an intrinsic ligand for the $\beta 2I$ domain and participates in the conformational activation of the integrin receptor (Alonso *et al*, 2002; Shimaoka *et al*, 2003a; Yang *et al*, 2004). In $\alpha L\beta 2$ integrin mutation E310A has been reported to push the equilibrium between the bent and extended conformations towards the bent conformation (Salas *et al*, 2004). In the $\alpha 2$ integrin, glutamate 336 (E336) in the $\alpha 7$ helix of the αI domain seems to have a similar role (Connors *et al*, 2007), as mutation E336A affects the activation of $\alpha 2\beta 1$ and the regulation of $\alpha 2I$ domain conformation (Connors *et al*, 2007). Thus $\alpha 2\beta 1$ integrin that harbours E336A mutation is in the bent rather than extended conformation. We report here that EV1, unlike any currently known extracellular matrix ligand, favours binding to the closed $\alpha 2I$ domain and inactive $\alpha 2\beta 1$ integrin. Furthermore, the activation of protein kinase C α (PKC α) and the EV1 entry pathway are independent of E336.

We have also studied structural requirements of $\alpha 2\beta 1$ signalling through p38 mitogen-activated protein kinase (MAPK) pathway, a signalling pathway strongly linked to $\alpha 2\beta 1$ integrin (Ivaska *et al*, 1999; Ravanti *et al*, 1999; Xu *et al*, 2001; Bix *et al*, 2004; Mazharian *et al*, 2005). Our results indicate that clustering, mediated either by collagen or antibodies, leads to rapid and transient activation of p38 MAPK. We also demonstrate that E336 in the $\alpha 2I$ domain is a key determinant in the $\alpha 2\beta 1$ -mediated activation of p38. Thus, the activation of p38 pathway can be considered as an indicator of E336-dependent conformational change in $\alpha 2\beta 1$ integrin. However, the clustering of $\alpha 2\beta 1$ integrin by EV1 did not significantly activate the p38 pathway during the early stage of infection. Thus, our results demonstrate that there seem to be fundamental differences in the mechanisms of EV1 action when compared with the natural ligands of $\alpha 2\beta 1$ integrin. EV1 seems to gain its entry by activation of signalling pathways that are dependent on $\alpha 2\beta 1$ clustering, but do not require conformational activation of the integrin.

Results

EV1 binds to the closed conformation of the $\alpha 2I$ domain

To compare the possible interactions of the open and closed conformations of $\alpha 2I$ with EV1, we constructed a model of the $\alpha 2I$ (open)–EV1 complex by superimposing the open-form crystal structure on our previously published model of the $\alpha 2I$ (closed)–EV1 complex. The model is based on a cryoelectron microscopy (cryo-EM) structure of the complex, into which the high-resolution crystal structures of EV1 and

the closed form of $\alpha 2I$ have been fitted (Xing *et al*, 2004). EV1 is known to bind in a metal ion-independent manner (Bergelson *et al*, 1993) at a binding site different from the MIDAS (King *et al*, 1997). The site has also been mapped by mutagenesis (King *et al*, 1997; Dickeson *et al*, 1999) to the side-face of the αI domain as opposed to the MIDAS location on top of the domain (Figure 1A). The comparison of the open and closed conformations shows that most of the region participating in virus binding on the αI domain surface is not involved in the closed–open conformational alteration and remains essentially unchanged (Figure 1A). The $\alpha 7$ helix that undergoes a large movement is located on the opposite side of the αI domain, compared with the virus-binding surface. Most of the loop between the $\beta 7$ strand and the $\alpha 6$ helix, including the αC helix in the closed conformation, is also not in close contact with EV1. Thus, the structural model can be considered to be compatible with EV1 binding to either the closed or the open conformation of the $\alpha 2I$ domain.

Previously, residue Asn289 in the αC helix of $\alpha 2I$ has been shown by mutagenesis to be required for EV1 binding (Dickeson *et al*, 1999). Here, in the modelled complexes (Figure 1A), this amino acid is in contact with the virus, but it is located at the periphery of the $\alpha 2I$ –EV1 interface and part of the residue is exposed to solvent. Asn289 is in the middle of a segment that undergoes an extensive structural rearrangement upon the $\alpha 2I$ change from the closed to open form, and therefore, it is possible to hypothesize that EV1 binding to Asn289 may affect the avidity or may even activate a conformational effect in the αI domain (Xing *et al*, 2004).

The introduction of the mutation E318W into $\alpha 2I$ has previously been shown to lead to a shift from the closed to the high affinity state open conformation (Aquilina *et al*, 2002; Tulla *et al*, 2008). Glu318 is located in helix $\alpha 7$, and it is not in contact with EV1 (Figure 1A). Here, the binding of human recombinant $\alpha 2I$ domains ($\alpha 2I$ WT and $\alpha 2I$ E318W) to EV1 was tested in a solid phase binding assay using microtitre plates coated with either the virus or collagen I as a control. The results were fitted to a Michaelis–Menten form equation and approximate K_d values were determined to quantify $\alpha 2I$ domain binding (Figures 1B and C). As expected, the mutation E318W increased $\alpha 2I$ domain binding to collagen I from $K_d \approx 39 \pm 3.5$ nM ($\alpha 2I$ WT) to $K_d \approx 7 \pm 0.5$ nM ($\alpha 2I$ E318W; Figure 1B). Wild-type $\alpha 2I$ bound tighter to EV1 than to collagen I in accordance with our previously published results (Xing *et al*, 2004). Surprisingly, the approximate K_d for $\alpha 2IE318W$ domain binding to EV1 seemed to be weaker ($K_d \approx 3 \pm 0.2$ nM) than the binding of wild-type $\alpha 2I$ ($K_d \approx 0.8 \pm 0.2$ nM; Figure 1C). Thus, the requirements for EV1 binding seem to differ from all previously tested $\alpha 2\beta 1$ integrin ligands that have been shown to favour the open $\alpha 2I$ conformation.

To further study the virus–receptor interaction additional experiments were performed. For BIAcore surface plasmon resonance measurements, EV1 was covalently coupled on the surface of the sensor chip. The measurements indicated that the association of both $\alpha 2I$ WT and $\alpha 2I$ E318W to immobilized EV1 was very fast (Figure 1D). Importantly, the dissociation of $\alpha 2I$ WT was remarkably slower when compared with $\alpha 2IE318W$ (Figure 1D), which partially explains the tighter binding of $\alpha 2I$ WT to EV1 seen in solid phase binding assays.

We also used BIAcore to perform kinetic titration series, in which samples were injected sequentially without a regen-

Figure 1 EV1 prefers the closed conformation of the $\alpha 2 \text{I}$ domain. (A) The crystal structure of the $\alpha 2 \text{I}$ domain in the closed (left) and the open conformations (right). Both conformations are also shown docked onto the surface of EV1 on the basis of the structure of the $\alpha 2 \text{I}$ –EV1 complex determined by cryo-EM (bottom). The surfaces of two protomers of the EV1 capsid are shown, one coloured light blue and the other light grey. The fivefold symmetry axis of the icosahedral capsid is labelled '5'. The regions in $\alpha 2 \text{I}$ that undergo the most extensive conformational changes (grey arrows) are the $\alpha 7$ helix (green), the βE – $\alpha 6$ loop (orange; includes the αC helix in the closed conformation) and the MIDAS (the metal ion in yellow). The position of amino-acid E318 is indicated. Residues that have been shown by mutagenesis to affect EV1 binding are coloured red (residues 199–201, 212–216 and 289), and the residues that are within 4.0 Å of the virus structure in the model of the complex are coloured blue. Most of the regions of the $\alpha 2 \text{I}$ involved in the conformational changes are not in close contact with the virus. Microtitre plates were coated with (B) collagen I (Col I) or (C) EV1. Delfia[®] Diluent II (BSA; Δ) was used as a background control. The GST fusion proteins, containing wild-type $\alpha 2 \text{I}$ (■) or high affinity mutant $\alpha 2 \text{I}$ E318W (O) domains, were allowed to react with the ligand for 1 h in the presence of 2 mM MgCl_2 . Bound αI domains were detected with Eu^{3+} -labelled GST antibody, and the signal was measured using time-resolved fluorescence. Data are presented as mean values $\pm$ s.d. of triplicate measurements. Approximate K_d values for αI domain binding were obtained by fitting the binding data for αI domain concentrations series to a Michaelis–Menten form equation. (D) BIAcore analyses of the interactions between 1 μM $\alpha 2 \text{I}$ WT (solid line) or $\alpha 2 \text{I}$ E318W (dashed line) with immobilized EV1 are presented as overlaid sensograms. After a short (120 s) association phase, the measurement of the dissociation was continued for 25 min. (E, F) Binding of $\alpha 2 \text{I}$ WT (■) and $\alpha 2 \text{I}$ E318W (O) to immobilized EV1 was competed with increasing concentrations of soluble (E) EV1 (0.04–0.7 nM) or (F) triple helical GFOGER peptide (0.01–1000 μM) that mimic a high-affinity integrin-binding site on collagen by using Eu^{3+} -based solid phase binding assay described above (B, C). Results were fitted to the model representing a dose–response curve with variable Hill slope.

eration step. The results failed to fit 1:1 binding model (Karlsson *et al*, 2006), indicating that more than one kind of binding site exist in EV1 (data not shown). To make further estimates of the stoichiometry of the virus binding to $\alpha 2 \text{I}$, increasing concentrations (0.04–0.7 nM) of soluble EV1 were allowed to compete with 1.5 nM $\alpha 2 \text{I}$ WT in binding to immobilized EV1 in a solid phase binding assay (Figure 1E). In two independent experiments, 0.17–0.34 nM EV1 seemed to block $\alpha 2 \text{I}$ binding to immobilized EV1. On the

basis of calculations using estimated M_r of virion (5.65×10^6) and $\alpha 2 \text{I}$ domain–GST fusion (49 500), the results indicated that in these conditions 7–15% of 60 putative integrin-binding sites per virion were occupied.

On the basis of molecular modelling, we have previously proposed that collagen and EV1 cannot concomitantly bind to $\alpha 2 \text{I}$ domain (Xing *et al*, 2004). To test this experimentally, a soluble triple helical GFOGER peptide, which mimics a high-affinity integrin-binding site in collagen (Knight *et al*, 1998), was

used in the competition study. Interestingly, the collagenous peptide (0.01–1000 μ M) effectively inhibited the binding of open $\alpha 2$ I (E318W) to EV1 (Figure 1F), but could not compete with the binding of closed $\alpha 2$ I (WT) to EV1 within the concentration range used. The result suggests that in the presence of collagen, EV1 significantly benefits from its preference for inactive $\alpha 2\beta 1$.

To test the phenomenon at the cellular level, Chinese hamster ovary (CHO) cells expressing either full-length $\alpha 2$ WT or $\alpha 2$ E318W were allowed to attach to immobilized collagen I, EV1 or BSA for 15 min. CHO cells do not naturally express integrin-type collagen receptors (Nykqvist *et al*, 2000). Adherent cells were detected using WST-1 reagent. In agreement with the experiments performed at the $\alpha 2$ I domain level, integrin activation significantly ($P < 0.001$) increased cell adhesion to collagen I (Figure 2). At the same time the activation decreased cell adhesion to EV1 ($P < 0.05$; Figure 2). Thus, EV1 seemed to bind better to the closed than open $\alpha 2$ I conformation and not only at the $\alpha 2$ I domain level but also when the tests are performed using full-length $\alpha 2$ integrins expressed on cell surface.

EV1 favours inactive $\alpha 2\beta 1$ integrin

To study further the structural requirements of EV1 binding to $\alpha 2\beta 1$ integrin, we constructed a conformationally inactive integrin and expressed it on cell surface. We have used molecular modelling to assess, whether the $\alpha 2$ I domain could interact with the $\beta 1$ subunit in the same manner as α LI and α MI interact with the $\beta 2$ subunit (Alonso *et al*, 2002; Shimaoka *et al*, 2003b; Yang *et al*, 2004; Arnaout *et al*, 2005). Our model suggests that when the $\alpha 2$ I domain is in the open conformation, E336 could interact with the metal ion of MIDAS of the $\beta 1$ I domain (Connors *et al*, 2007; Figure 3A). The model predicts that collagen binding to the $\alpha 2$ I domain will most probably induce a conformational change in the $\beta 1$ I domain that leads to the separation of the integrin leg

Figure 2 Integrin activation decreased EV1 binding to cellular $\alpha 2\beta 1$. CHO- $\alpha 2$ and CHO- $\alpha 2$ E318W cells were allowed to adhere to immobilized EV1 or collagen I for 15 min. Adherent cells were detected using WST-1 reagent. Integrin activation significantly ($P < 0.001$) increased cell adhesion to collagen I. At the same time, EV1 seemed to favour CHO- $\alpha 2$ WT cells ($P < 0.05$). Mean values $\pm$ s.d. of eight parallel measurements are shown. Statistical significances were determined by two-tailed Student's *t*-test.

regions, as has been described for other integrins (Alonso *et al*, 2002; Kim *et al*, 2003; Yang *et al*, 2004). In addition to E336 at the C-terminal end of helix $\alpha 7$, E309 in the loop preceding helix $\alpha 7$ was chosen as a target for mutagenesis (Figure 3A).

Wild-type $\alpha 2$ and mutant $\alpha 2$ integrins, containing either E336A or E309A, were expressed in CHO cells. Equal expression levels of the mutant $\alpha 2$ integrins were confirmed by flow cytometry (Supplementary Figure S1). In addition, cell lines were metabolically labelled with 35 S-methionine/cysteine, and the $\alpha 2\beta 1$ complex was immunoprecipitated with $\alpha 2$ antibodies under conditions that maintained the subunit interactions. Analysis by SDS-PAGE confirmed that the expression levels of the mutant integrins were practically equal. Furthermore, the mutations seemed to have no effect on the stability of the $\alpha 2\beta 1$ heterodimer (data not shown). When tested by a spreading assay on collagen I, CHO- $\alpha 2$ E309A cells did not significantly differ from CHO- $\alpha 2$ cells expressing the wild-type $\alpha 2$ subunit (Figure 3B). Cells carrying the point mutation, E336A, in their $\alpha 2$ subunit were able to attach to collagen I but their spreading was delayed. Similar results were obtained when the CHO- $\alpha 2$ and CHO- $\alpha 2$ E336A cells were allowed to attach to an immobilized collagen I for 15 min and adherent cells were detected with WST-1 reagent (Figure 3C). The results suggest that the mechanism of $\alpha 2\beta 1$ action would closely resemble that of α L $\beta 2$ integrin (Yang *et al*, 2004): the communication of the conserved residue E336 in $\alpha 2$ with the metal ion at MIDAS of the $\beta 1$ I domain may induce the high-affinity conformation of the $\alpha 2$ I domain. Importantly, when CHO- $\alpha 2$ and CHO- $\alpha 2$ E336A binding to EV1 was analysed in the adhesion assay, EV1 seemed to favour the inactive state of $\alpha 2\beta 1$ (Figure 3C). Thus, the results with the E336A variant confirmed the idea that EV1 binds to inactive rather than active integrins. Furthermore, the corresponding mutation in α L integrin has been reported to push the equilibrium between the bent and extended conformations towards the bent conformation (Salas *et al*, 2004). Therefore, the data propose that EV1 may bind to bent rather than extended integrins.

We have previously shown that activation of PKC by TPA induces both ligand-independent macroaggregation of $\alpha 2\beta 1$ integrins and conformational activation of $\alpha 2$ I, whereas the E336A mutation prevents the change in conformation but not receptor clustering (Connors *et al*, 2007). When TPA-treated (100 nM) cells were allowed to attach to collagen I for 15 min, both CHO- $\alpha 2$ and CHO- $\alpha 2$ E336A cell adhesions were increased (Figure 3D). Similarly, TPA increased binding of EV1 to the $\alpha 2$ WT and the $\alpha 2$ E336A mutant cells. A previous study has also shown that TPA can increase EV1 binding to $\alpha 2\beta 1$ integrin (Bergelson *et al*, 1993). Our observations indicate that the formation of $\alpha 2\beta 1$ clusters before ligand binding, rather than the conformational activation of the integrin, may explain the increased integrin avidity to EV1.

Activation of p38 after $\alpha 2\beta 1$ integrin clustering by collagen I requires E336-dependent conformational changes in the integrin

Next we tested the structural requirements of $\alpha 2\beta 1$ signalling after integrin clustering. The formation of $\alpha 2\beta 1$ integrin clusters after treatment with $\alpha 2$ subunit-specific primary antibodies and clustering secondary antibodies was imaged

Figure 3 EV1 favours inactive $\alpha 2 \beta 1$ integrin. (A) A structural model of the human $\alpha 2 \beta 1$ integrin head region was built based on the crystal structures of the $\alpha V \beta 3$ integrin and the $\alpha 2$ domain. (A; bottom, left) Modelling indicates that the closed conformation of the $\alpha 2$ domain does not form specific contacts with the $\beta 1$ domain (blue). (A; bottom, right) However, when the $\alpha 2$ domain adopts an open conformation, E336 at the C-terminal end of helix $\alpha 7$ is positioned in such a way that it could coordinate to the metal ion (yellow) of the MIDAS in the $\beta 1$ domain and act as an intrinsic ligand. E309 does not seem to participate in the process. (B) In a cell spreading assay, CHO- $\alpha 2$ and CHO- $\alpha 2$ E336A cells attached and spread on a collagen I (Col I) matrix in 120 min, whereas $\alpha 2$ E336A mutation caused a dramatic decrease in the spreading. Cells were not able to attach to or spread on BSA, which was used as a negative control. (C) When CHO- $\alpha 2$ and CHO- $\alpha 2$ E336A cells were allowed to adhere to immobilized EV1 or collagen I for 15 min, the E336A mutation seemed to decrease $\alpha 2$ -mediated cell adhesion to collagen I. Interestingly, EV1 seemed to favour CHO- $\alpha 2$ E336A cells. Mean values $\pm$ s.d. of four parallel measurements are shown (B, C). (D) However, at 15 min, CHO- $\alpha 2$ and CHO- $\alpha 2$ E336A cell adhesion to both collagen I and EV1 was significantly increased in the presence of integrin cluster-inducing TPA (100 nM; black columns). Mean values $\pm$ s.d. of four parallel measurements are shown.

using confocal microscopy. At 15 min, clear integrin clusters appeared when primary and secondary antibodies were used together (Figure 4A). Collagen binding to $\alpha 2 \beta 1$ has been reported to lead to specific activation of the p38 α MAPK signalling pathway (Ivaska *et al*, 1999; Ravanti *et al*, 1999; Xu *et al*, 2001; Bix *et al*, 2004; Mazharian *et al*, 2005). To analyse the effect of $\alpha 2 \beta 1$ cluster formation on p38 activation, Saos- $\alpha 2$ and CHO- $\alpha 2$ cells were treated with the antibodies and p38 phosphorylation was analysed by immunoblotting. Antibody-induced clustering caused a rapid and transient phosphorylation of p38 at 15 min in both cell lines (Figures 4B and C). The transient nature of p38 activation (Figure 4B)

is most probably because of the fact that antibody-generated $\alpha 2 \beta 1$ clusters are rapidly internalized (Upla *et al*, 2004). To confirm the observation, we also analysed the phosphorylation of p38 by a flow cytometry-based method and a similar activation of p38 in CHO- $\alpha 2$ cells was detected (Figure 4D). It was also obvious that neither the $\alpha 2$ integrin-specific antibody, nor the secondary antibody alone affected p38 phosphorylation (Figure 4D).

To analyse the effect of inactivating mutation E336A on $\alpha 2 \beta 1$ integrin signalling, we performed two series of experiments. CHO- $\alpha 2$ and CHO- $\alpha 2$ E336A cells were exposed either to collagen I or fibronectin immobilized on cell culture

Figure 4 Clustering of $\alpha 2 \beta 1$ integrins induces a rapid transient phosphorylation of p38. (A, bottom) Volume renderings of confocal image data show that secondary antibodies (goat anti-mouse IgG) were able to induce integrin clustering in Saos- $\alpha 2$ cells treated with $\alpha 2$ primary antibody (Alexa Fluor 555-conjugated 16B4). (A, top) Without secondary antibodies, no clustering occurs. In both cases, the same living cell was imaged at 0- and 15-min time points. (B) When p38 phosphorylation (P-p38) induced by the antibody (16B4 and anti-mouse IgG)-mediated $\alpha 2 \beta 1$ clustering was analysed in Saos- $\alpha 2$ cells at successive time points, a rapid and transient p38 phosphorylation, peaking at 15 min, was obvious. (C) Similarly, antibody-mediated clustering caused p38 activation even in CHO- $\alpha 2$ cells in 15 min. Representative immunoblots of one experiment (B, C) and statistical analyses of scanned blot images of one (C) or five (B) independent experiments are shown. Mean levels of phosphorylated p38 (P-p38) $\pm$ s.d. relative to total p38 or β -actin levels are shown. (D) In addition to immunoblotting, flow cytometric analysis of Saos- $\alpha 2$ cells showed that neither $\alpha 2$ -specific primary nor secondary antibody alone caused p38 activation, whereas the combination of the antibodies elicited p38 phosphorylation. For analysis, cells were treated with clustering antibodies for 15 min, fixed with isopropanol, permeabilized with methanol and stained with Alexa Fluor 488-conjugated phospho-p38 antibody.

plates (Figure 5A and B). When p38 phosphorylation was measured by immunoblotting, it seemed that CHO- $\alpha 2$ cells contained higher phospho-p38 levels on collagen I than on fibronectin (Figure 5A). However, in the CHO- $\alpha 2$ E336A cells, p38 was not phosphorylated on collagen I, whereas its activation was clear on fibronectin (Figure 5A). Alternatively, $\alpha 2$ antibodies were used to cluster integrins (Figure 5B and C). In both CHO- $\alpha 2$ (Figure 5B) and Saos- $\alpha 2$ (Figure 5C) cells, p38 activation was obvious, whereas the E336A mutation inhibited the effect. On the basis of these data, in the following experiments, we used p38 activation as an indicator of E336-dependent conformational change in $\alpha 2 \beta 1$ integrin.

EV1 entry is independent of E336-mediated conformational activation of $\alpha 2 \beta 1$

In addition to clustering antibodies, a similar relocation of $\alpha 2 \beta 1$ integrins to macroaggregates on the Saos- $\alpha 2$ cell surface was induced by EV1 after 15 min (Figure 6A). Both EV1 and antibody treatments caused similar cluster formation even in Saos- $\alpha 2$ E336A cells (data not shown).

We have shown above that p38 activation after antibody-mediated clustering requires E336-dependent conformational changes in the integrin. This indicates that the clusters of extended $\alpha 2 \beta 1$ integrins activate different signalling pathways when compared with bent integrins. Accordingly, $\alpha 2 \beta 1$ clustering caused by EV1 did not induce p38 activation (Figure 6B), suggesting that binding of the virus to $\alpha 2 \beta 1$ does not lead to the activation of integrin conformation. Previously, EV1 entry has been shown to require PKC α activation (Upla *et al*, 2004). After 30-min EV1 clustering, both Saos- $\alpha 2$ and Saos- $\alpha 2$ E336A cells showed similar PKC α activation when analysed by immunoblotting using phospho-specific antibody (Figure 6C). Finally, we imaged the ability of EV1 to infect cells that express mutant $\alpha 2 \beta 1$ integrins using confocal microscopy. Similarly to PKC α activation, even EV1 infection was observed both in Saos- $\alpha 2$ and Saos- $\alpha 2$ E336A cells (Figure 6D). The results indicate that EV1 neither activates $\alpha 2 \beta 1$ in the same manner as the natural ligands, nor is dependent on these activation mechanisms during its cell-entry process.

EV1 may bind to integrins that are in the bent conformation

Our observations indicate that EV1 can bind to $\alpha 2 \beta 1$ integrins that have closed $\alpha 2$ I domains, and also to integrins that are unable to effectively interact with natural ligands as a consequence of E336A mutation. Furthermore, the integrins harbouring E336A mutations may take the bent conformation. To test this further, we allowed CHO- $\alpha 2$ cells to adhere to collagen I or EV1 for 15 min in the presence of 2 mM ethylenediamine tetraacetate (EDTA). EDTA prevented cell adhesion to collagen I, but not to EV1 (Supplementary Figure S2A). The result replicates an old observation that EV1 can bind to $\alpha 2 \beta 1$ integrin in the absence of divalent cations (Bergelson *et al*, 1993) and reflects the fact that EV1 binding, unlike collagen binding, is independent of Mg^{2+} ion. However, the data also support the hypothesis that EV1 binds to the bent rather than extended $\alpha 2 \beta 1$ integrin, as more recent observations, based on activation-dependent antibodies, indicate that EDTA keeps integrins in the bent conformation (Xie *et al*, 2004). Furthermore, we could show that 2 mM EDTA also prevents p38 activation after $\alpha 2 \beta 1$ clustering, supporting the essential role of extended conformation in the process (Supplementary Figure S2B).

To further test the hypothesis that EV1 binds to the bent $\alpha 2 \beta 1$ integrin, we constructed models of the $\alpha 2 \beta 1$ heterodimer bound to EV1 in the bent and the extended conformation (Figure 7). The icosahedral EV1 capsid consists of 60 identical copies of the capsid protomer. Five protomers are arranged symmetrically in a pentameric structure around a fivefold symmetry axis at each of the 12 vertices of the icosahedron. Each protomer contains one binding site for the $\alpha 2$ I domain. We have previously published a model in which the $\alpha 2 \beta 1$ ectodomain in the extended conformation can occupy all of the five sites in one pentamer on the EV1

Figure 5 Activation of p38 after $\alpha 2 \beta 1$ clustering by antibodies or natural ligands requires E336-dependent conformational changes in $\alpha 2$ subunit. (A) CHO- $\alpha 2$ and CHO- $\alpha 2$ E336A cells were sub-cultured onto collagen I (Col I) or fibronectin-coated cell culture plates. After o/n incubation, cells were lysed and p38 phosphorylation (P-p38) was detected by immunoblotting. CHO- $\alpha 2$ cells showed higher p38 activation on collagen I than on fibronectin. Fibronectin seemed to activate p38 even in CHO- $\alpha 2$ E336A cells; however, the E336A mutation inhibited $\alpha 2 \beta 1$ integrin-mediated p38 activation induced by collagen I. (B, C) Similarly, E336A caused a dramatic decrease in p38 activation after antibody-mediated $\alpha 2$ clustering both in (B) CHO- $\alpha 2$ E336A and (C) Saos- $\alpha 2$ E336A cells, whereas the p38 phosphorylation was induced in CHO and Saos cells expressing $\alpha 2$ WT. Immunoblots and quantified data from representative experiments are shown. The mean level of p38 activation is shown relative to (A) total p38 or (B, C) β -actin levels.

surface without steric hindrance (Xing *et al*, 2004; Figure 7A). In the case of the bent conformation, a much wider receptor structure must be accommodated close to the virus surface. However, the linker segments connecting the $\alpha 2$ I domain to the propeller domain and the ectodomain to the transmembrane helices are long and flexible enough to allow the heterodimer to adopt a large range of different conformations. We constructed a realistic model of a bent heterodimer that can fit into adjacent binding sites in one EV1 capsid pentamer (Figure 7B). However, the five integrins are necessarily packed very closely together and against the membrane in this arrangement, and the orientation of the $\alpha 2$ I domain relative to the rest of the ectodomain is substantially different from that in the extended conformation model. An alternative model, in which integrins, in the bent conformation, are located at larger distances from each other around the virus surface, can also be constructed (Figure 7C and D). In this case, the $\alpha 2 \beta 1$ heterodimers are bound to protomers in different, adjacent pentamers. The transmembrane helices of the five heterodimers point roughly in the same direction, but the model requires the cell membrane to be curved around the viral particle. The illustrated models represent idealized cases in which each binding site of a symmetric set is occupied. On the basis of the results shown in Figure 1E, up to 10 out of 60 putative integrin-binding sites can be concomitantly occupied. This stoichiometry supports the models presented in Figures 7C and D rather than the model in Figure 7B.

Discussion

EV1 is a human pathogen, which belongs to the *Picornaviridae* family of RNA viruses and causes meningoencephalitis, carditis and rashes, as well as mild respiratory and enteric diseases (Grist *et al*, 1978). Earlier studies have shown that EV1 binds to $\alpha 2 \beta 1$ on the cell surface and that the life cycle of this virus is critically dependent on the receptor (Bergelson *et al*, 1992, 1993). This has been evident in experiments performed on human cell lines, such as Saos-2 osteosarcoma cells that are $\alpha 2 \beta 1$ integrin negative and resistant to EV1 infection, but can act as EV1 host cells after transfection with $\alpha 2$ integrin cDNA (Marjomäki *et al*, 2002). Despite the facts that EV1 cannot infect mouse $\alpha 2 \beta 1$ integrin-expressing cell lines and that mouse integrin $\alpha 2$ I domain does not bind to EV1 (Bergelson *et al*, 1994; Zhang and Racaniello, 1997), a transgenic mouse harbouring human $\alpha 2$ integrin is susceptible to the infection (Hughes *et al*, 2003). Taken together, the data suggest that high-affinity recognition of a cellular receptor is an essential step in the EV1 infection.

In addition to receptor binding, activation of an entry pathway is an essential process in the life cycle of several viruses. Many viruses that use members of the integrin family as their cellular receptors are internalized in clathrin-coated pits and later found in endosomes (Wickham *et al*, 1993; Joki-Korpela *et al*, 2001; Jin *et al*, 2002; O'Donnell *et al*, 2005). However, EV1 forms an exception, as, inside the host

Figure 6 $\alpha 2 \beta 1$ clustering, not the activation of p38 or E336-mediated conformational activation of the receptor, is required for the EV1 entry. (A) In Saos- $\alpha 2$ cells EV1 induced cluster formation of $\alpha 2$ integrins in 15 min as indicated by the co-localization image (scale bar, 10 μ m). Manders' co-localization coefficients are 0.52 for integrin and 0.51 for EV1 and $P = 1.0$. (B) A 15-min EV1 clustering does not, however, induce p38 activation similar to that seen in antibody-mediated clustering in Saos- $\alpha 2$ cells (C). In both Saos- $\alpha 2$ and Saos- $\alpha 2$ E336A cells, 30-min EV1 clustering induced a clear PKC α activation. (B, C) An immunoblot and quantified data (mean $\pm$ s.e.) of five or three representative experiments are shown. Untreated cells or cells treated with $\alpha 2$ primary antibody (16B4) only were used as controls. (D) When the ability of EV1 to infect Saos- $\alpha 2$ and Saos- $\alpha 2$ E336A was studied, both EV1 and $\alpha 2 \beta 1$ were labelled, and EV1-positive cells were manually calculated from confocal microscopy images. The E336A mutation introduced into $\alpha 2$ subunit seemed not to prevent EV1 entry into Saos cells. Data shown are mean values $\pm$ s.d. of EV1-positive cells (%).

cell, virus particles seem to accumulate in caveolin-1-positive structures (Marjomäki *et al*, 2002). On the host cell surface, some EV1 particles can be detected inside caveolae (Marjomäki *et al*, 2002), but other entry mechanisms may be more important (Pietiäinen *et al*, 2004). We have recently

proposed that EV1 is internalized through a clathrin- and caveolin-independent mechanism that resembles macropinocytosis (Karjalainen *et al*, 2008). After binding to $\alpha 2 \beta 1$ integrin, EV1 has been shown to activate PKC α and inhibition of this signalling pathway also blocks the entry process (Upla *et al*, 2004). Other integrin-binding viruses are also known to activate various signalling proteins. For example, binding of HHV-8 to $\alpha 3 \beta 1$ integrin activates the focal adhesion kinase immediately downstream in the outside-in signalling pathway mediated by integrins, this leads to further activation of several signalling molecules (Krishnan *et al*, 2006). In addition, the endocytosis of human adenoviruses that recognize αV integrins is dependent on the activation of phosphoinositide-3-OH kinase (Li *et al*, 1998). New approaches, such as the use of siRNA silencing, will most probably cast new light on the complex signalling pathways involved in virus internalization (Pelkmans *et al*, 2005).

In general, viruses are regarded as mimicking natural integrin ligands in their receptor-binding mechanism, as well as in the activation of cellular signalling. Human adenovirus type 2 (Ad2) is a good example. Cryo-EM has shown that the integrin-binding RGD protrusion of the Ad2 penton base protein is highly mobile (Stewart *et al*, 1997), in the same manner as the RGD motif in fibronectin (Main *et al*, 1992). When the structure of soluble recombinant integrin $\alpha V \beta 5$ in complex with Ad2 was determined by cryo-EM, the results suggested that the precise spatial arrangement of five RGD protrusions on the penton base promotes integrin clustering and the signalling events required for virus internalization (Chiu *et al*, 1999).

When compared with Ad2, our experiments using EV1 and $\alpha 2 \beta 1$ integrin give a very different idea about the virus-integrin interaction. In αI domain-containing integrins, the binding of a natural ligand, such as collagen, triggers a change from the closed to the open αI domain conformation (Emsley *et al*, 2000). We tested EV1 binding to $\alpha 2 I$ domain harbouring the E318W mutation, which allows the integrin $\alpha 2 I$ domain to adopt the open high-affinity conformation. In contrast, the wild-type $\alpha 2 I$ domain has a salt bridge between residues R288 and E318, stabilizing the closed low-affinity state (Aquilina *et al*, 2002; Tulla *et al*, 2008). Unexpectedly, we noticed that EV1, unlike any other $\alpha 2 \beta 1$ ligand, favours the closed $\alpha 2 I$ domain over the open state. The result was confirmed using CHO cells expressing full-length wild-type $\alpha 2$ or $\alpha 2$ E318W integrin. To further study the phenomenon in cell culture, we used cells transfected to express $\alpha 2$ E336A mutant integrin. We have previously shown that $\alpha 2 \beta 1$ integrin avidity to collagen I is regulated by two synergistic mechanisms: first, an $\alpha 2$ E336-dependent switch to the open $\alpha 2 I$ conformation; second, an $\alpha 2$ E336-independent mechanism associated with receptor aggregation (Connors *et al*, 2007). Previous studies have also indicated that mutation of the residue corresponding to $\alpha 2$ E336 in αM (E320; Alonso *et al*, 2002) and αL (E310; Huth *et al*, 2000) may completely inactivate the integrin. In agreement with the previous observations (Connors *et al*, 2007), CHO- $\alpha 2$ E336A cells could attach to collagen I but their spreading was significantly delayed. CHO- $\alpha 2$ cells were also able to adhere to immobilized collagen I better than the mutant CHO- $\alpha 2$ E336A cells. Despite the fact that the mutation E336A in the $\alpha 2$ subunit prevents the conformational activation of $\alpha 2 \beta 1$ integrin and decreases cell adhesion to collagen I, the muta-

Figure 7 Structural models of the binding of $\alpha 2 \beta 1$ integrin clusters to EV1. (A) Five $\alpha 2 \beta 1$ heterodimers (different shades of orange and red) in the extended conformation are shown bound to the five EV1 capsid (light blue) protomers around one fivefold symmetry axis. $\alpha 2$ I domains are drawn in green and the transmembrane helices in dark green. The position of the membrane is marked with a grey bar. The position and orientation of the unoccupied $\alpha 2$ I domain-binding sites on the virus surface (one in each of the 60 protomers) is marked with green L-shaped lines. (B) Five $\alpha 2 \beta 1$ heterodimers in the bent conformation are shown bound to five protomers (forming a pentamer) around a fivefold symmetry axis. This arrangement results in the five integrins packing very closely together and against the membrane, but it may still be sterically feasible. The $\alpha 2$ I domain and the transmembrane helices are connected to the rest of the integrin structure by highly flexible linkers that may allow a bent conformation that can fit in the neighbouring binding sites of one pentamer. (C) Five $\alpha 2 \beta 1$ heterodimers in the bent conformation, each bound to one protomer in five adjacent pentamers around the EV1 capsid. A grey line marks the position of the plasma membrane. The transmembrane helices of the five integrins are positioned such that the membrane would be required to be substantially curved around the virus. The same arrangement, looking down an axis perpendicular to the membrane, is shown in (D). In this pentagonal arrangement, the distance from the intracellular end of one transmembrane helix to corresponding part in the proximal, neighbouring integrin is approximately 320 Å and to a distal integrin is approximately 500 Å.

tion increased adhesion to EV1. Thus, experiments using recombinant α I domains and cell lines harbouring integrin mutations led to the conclusion that EV1, unlike the natural ligands of $\alpha 2 \beta 1$ integrin, has evolved to recognize the non-activated integrin conformation. These observations, together with the fact that EV1 binding to $\alpha 2 \beta 1$ integrin also takes place in the absence of divalent cations, suggest that EV1 may also bind to integrins in the bent conformation. The integrin ectodomain is highly flexible, and it may be possible to accommodate $\alpha 2 \beta 1$ heterodimers in the bent conformation at adjacent binding sites in the pentamer of the EV1 capsid, even though this leads to very close packing of the integrins. Our calculations on the basis of molar ratios of $\alpha 2$ I domain binding to a concentration series of EV1 suggest a stoichiometry in which up to 10 out of 60 putative integrin-binding sites can be occupied at the same time. This supports another model in which bent $\alpha 2 \beta 1$ heterodimers are bound at non-adjacent sites around the virus surface, assuming that the plasma membrane is curved around the viral particle. In this case, the intracellular domains of the integrins would be located about 320 Å apart, possibly forming a ring-

like adhesion site. As integrin signalling is mediated by proteins that bind to intracellular domains of integrins, the unique architecture of EV1-dependent integrin clusters may govern the entry mechanism and life cycle of EV1.

On the basis of our structural model of the $\alpha 2$ I–EV1 complex, we could not exclude the possibility that EV1 binding to the $\alpha 2$ I domain could induce a conformational change from the closed to open form. Therefore, we selected an indirect way to test whether the conformational change in $\alpha 2 \beta 1$ integrin takes place after EV1 binding. So far, we have been unable to use direct methods to monitor integrin conformation due to technical problems. Previously, the tail separation of the $\alpha L \beta 2$ heterodimer has been demonstrated by using integrins with fluorescent protein tags fused to their intracellular domains (Kim *et al*, 2003). However, we have not succeeded in expressing tagged $\alpha 2$ subunits in the cells that we used in these experiments. The indirect approach was based on the previous observation that $\alpha 2 \beta 1$ integrin specifically activates p38 MAPK (Ivaska *et al*, 1999). Here, we could show that antibody-mediated clustering of $\alpha 2 \beta 1$ integrin causes a rapid but transient phosphorylation of p38.

However, when the E336A mutation is introduced into the $\alpha 2$ subunit, the activation of p38 is not achieved. Thus, clustering itself is not sufficient for p38 activation but the conformational modulation of $\alpha 2\beta 1$ is also required. The observation also allowed us to use p38 phosphorylation as an indicator of the conformational activation of $\alpha 2\beta 1$ integrin after EV1-mediated clustering. Importantly, EV1-induced clustering did not evoke p38 activation at an early stage of infection, strongly suggesting that after virus binding $\alpha 2\beta 1$ does not undergo a change to similar conformation that takes place after clustering induced by either antibodies or natural ligands. In previous studies, p38 activation has been studied some hours after infection and p38 seems to be phosphorylated when EV1 replication has started (Huttunen *et al*, 1998). The E336A mutation in the $\alpha 2$ subunit did neither affect EV1 induced activation of PKC α , which has been previously linked to EV1 internalization (Upla *et al*, 2004), nor the ability of EV1 to infect cells. Thus, the results propose that EV1 does not activate its entry pathway by direct mimicry of natural ligands, but it has evolved to take the advantage of integrin biology in a unique manner.

To conclude, our observations during the early steps of the EV1 life cycle suggest that the virus binds first to inactive, bent $\alpha 2\beta 1$ integrins and clusters them without triggering a conformational activation of the receptor. Thus, EV1 seems to activate its own entry by simply clustering $\alpha 2\beta 1$ integrins. PKC α has been suggested to be one part of the entry machinery (Upla *et al*, 2004), and our new observations also indicate that PKC α activation by EV1 is not dependent on the E336-mediated conformational changes in $\alpha 2\beta 1$. Previous observations have shown that the deletion of the $\alpha 2$ cytoplasmic domain (Kawakuchi *et al*, 1994) or its replacement with the $\alpha 1$ cytoplasmic domain (Marjomäki *et al*, 2002) has no effect on EV1 entry. PKC α may interact with $\alpha 2\beta 1$ integrin through $\beta 1$ cytoplasmic domain (Connors *et al*, 2007), whereas the activation of p38 is dependent on the $\alpha 2$ cytoplasmic domain (Ivaska *et al*, 1999). Thus, EV1 also provides a unique tool to discriminate between the effects of clustering and conformational activation in integrin signalling, as well as between $\alpha 2$ - and $\beta 1$ -mediated signalling.

Materials and methods

EV1 and cell cultures

EV1 (Farouk strain, ATCC) was propagated in GMK cells and purified in sucrose gradients as previously described (Abraham and Colonna, 1984). Saos-2 (ATCC) and CHO cells (ATCC) were cultured and stably transfected with human $\alpha 2$ or with the $\alpha 2$ E336A, $\alpha 2$ E309A mutation in the pAWneo2 expression vector as described (Ivaska *et al*, 1999; Connors *et al*, 2007). A point mutation E318W was introduced into human $\alpha 2$ cDNA in a pcDNA 3.1 expression vector (Invitrogen) using QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. The construct was stably expressed in CHO cells using the previously described method (Connors *et al*, 2007).

Molecular modelling

Structural models of human $\alpha 2\beta 1$ headpiece domains were based on the crystal structures on the $\alpha V\beta 3$ and the $\alpha 2$ I domain as described (Connors *et al*, 2007). The model of the $\alpha 2$ I (closed)-EV1 complex, based on cryo-EM data and the crystal structures of EV1 (Filman *et al*, 1998; 1EV1) and $\alpha 2$ I domain in the closed conformation (Emsley *et al*, 1997; 1AOX), was constructed earlier by Xing *et al* (2004). The EV1 complex with the open conformation was built by superimposing on this model the open form I domain structure (1DZI; Emsley *et al*, 2000). To model clusters of $\alpha 2\beta 1$ bound to EV1,

a comparative model of the entire $\alpha 2\beta 1$ integrin heterodimer in the bent conformation was built with Modeller 9.6 (Marti-Renom *et al*, 2000) using the crystal structures of both $\alpha V\beta 3$ (1JV2; Xiong *et al*, 2001) and $\alpha 1\beta 3$ (Zhu *et al*, 2008; 3FCS) as templates. The crystal structure of $\alpha 2$ I in the closed conformation (1AOX) was added. The orientation of the I domain was modelled manually, constrained by the distance of $\alpha 2$ E336 to the β I domain MIDAS site. The extended conformation of the dimer was modelled by moving the domains by hand to match published electron micrographs. Molecular graphics in Figures 1A, 3A and 7 were created using PyMOL v1.1 (DeLano, 2002).

Solid phase binding assay

The $\alpha 2$ I domain was generated by PCR using human integrin $\alpha 2$ cDNA as a template (Ivaska *et al*, 1999). E318W point mutation was introduced into the $\alpha 2$ I domain as described previously (Tulla *et al*, 2008). Both $\alpha 2$ I WT and $\alpha 2$ I E318W were produced as GST fusion protein as previously described (Tulla *et al*, 2001). Microtitre plates (Costar) were coated with collagen I (20 μ g/cm²; PureCol, INAMED Biomaterials) or with similar concentrations of EV1 o/n at 4°C. In the competition studies, 1.5 nM $\alpha 2$ I domain was allowed to attach to immobilized EV1 in the presence of 0.04–0.7 nM EV1 or 0.01–1000 μ M GFOGER collagen peptide (Auspep, Australia) synthesized as previously described (Knight *et al*, 2000). Otherwise the assay was carried out essentially as previously described (Jokinen *et al*, 2004).

BIAcore analysis

Measurements were performed using surface plasmon resonance on a BIAcore-X (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). EV1 was covalently coupled through primary amine groups to the dextran matrix of a CM5 sensor chip (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using sodium formate (pH 3.0) according to Lea *et al* (1998). Bound EV1 levels were adjusted to about 2000 resonance units (RU). The 1 μ M $\alpha 2$ I WT and $\alpha 2$ I E318W were passed over the chip at flow rate 30 μ l/min at 25°C. HEPES-buffered saline (10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005 % surfactant P20; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used as a running buffer throughout.

Cell spreading and adhesion assay

For the assays, microtitre plates (Costar) were coated with collagen I (20 μ g/cm²), EV1 or 1 % BSA o/n at 4°C. The spreading assay was performed as described previously (Jokinen *et al*, 2004). In the adhesion assay, non-specific binding sites were blocked with 1 % BSA in PBS for 1 h at 37°C before addition of 2×10^5 cells per well. When indicated, cells were pre-treated with 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Calbiochem) in serum-free media for 10 min. EtOH was added to control cells. Cells were allowed to attach for 15 min at 37°C in the presence of 2 mM MgCl₂ or 2 mM EDTA. Wells were then washed and adherent cells were detected using tetrazolium salt WST-1 reagent (Roche) according to manufacturer's instructions. The absorbance was measured at 450 nm (Labsystems Multiscan Plus).

Confocal microscopy imaging of integrin clustering

Saos- $\alpha 2$ cells were cultured as described above, but on chambered cover glasses with CO₂-independent medium (Sigma). For antibody clustering, cells were incubated with Alexa-555-conjugated mAb against $\alpha 2$ integrin (16B4, Serotec) for 15 min at 37°C. The secondary antibody (goat anti-mouse IgG, Molecular Probes) was added to induce clustering. Three-dimensional image stacks of the same cell were obtained with a laser scanning confocal fluorescence microscope (Carl Zeiss, Axiovert 100 M with LSM510), before and 15 min after adding the secondary antibody. For the negative control no secondary antibody was added. Alternatively, Saos- $\alpha 2$ cells were incubated with EV1 for 15 min at 37°C, samples were fixed with 4 % PFA for 20 min at RT, and stained for EV1 and $\alpha 2$ integrin. To detect EV1, rabbit anti-EV1 (Marjomäki *et al*, 2002) and Alexa-555-conjugated secondary antibodies (Molecular Probes) were used. Integrin $\alpha 2$ was stained with mouse mAb 16B4 (Serotec) and Alexa-488-conjugated secondary antibody (Molecular Probes). Three-dimensional ray cast opacity volume renderings and co-localization analyses (automatic thresholding after background subtraction, Costes *P*-value calculation with 100 iterations) of selected image stacks were performed using BioImageXD software (Kankaanpää *et al*, 2006).

Measurement of EV1 infection

For the measurement of EV1 infection, Saos-WT, Saos- $\alpha 2$ and Saos- $\alpha 2E336A$ cells were incubated with EV1 for 2–6 h and fixed with 3% paraformaldehyde for 20 min at RT. Triton X-100 (0.1%) permeabilized cells were stained for EV1 and $\alpha 2$ integrin as mentioned above. Finally, cells were observed under confocal microscope (Carl Zeiss Axiovert 100 M with LSM510), and the percentage of EV1-positive cells was determined by counting manually.

p38 and PKC α activation induced by integrin clustering

Cells incubated with 0.1% FCS in DMEM o/n, were treated with integrin $\alpha 2$ antibody (16B4, Serotec) for 15 min at 37°C, followed by 15–120-min secondary antibody treatment (anti-mouse IgG, DAKO) at 37°C. Alternatively, cells were incubated with EV1 for 15 min (P-p38) or 30 min (P-PKC α) at 37°C. When indicated, clustering was induced in the presence of 2 mM EDTA. Finally, cells were collected and analysed by immunoblotting as described below.

p38 activation on collagen I and fibronectin-coated plates

Cell culture plates were coated with collagen I or with human plasma fibronectin (Chemicon International) 20 $\mu\text{g}/\text{cm}^2$ in PBS o/n at 4°C. Before addition of cells, the coated wells were washed with PBS and blocked with 0.1% BSA in PBS for 1 h at 37°C. Cells were allowed to attach in serum-free αMEM at 37°C, 5% CO_2 o/n. Finally, cells were collected and the samples were immunoblotted as described below.

Analysis of kinase activation

Cells were lysed in Laemmli SDS-PAGE sample buffer and the sonicated samples were separated on a 10% acrylamide SDS-PAGE gel and electroblotted onto a Hybond ECL membrane (Amersham, UK). Membranes were probed for activated p38 as previously described (Ivaska *et al*, 1999) using a phospho-specific p38 antibody (T180/Y182; Cell Signaling Technology or Zymed) and antibodies against total p38 (Cell Signaling Technology or Zymed)

or β -actin (I-19, Santa Cruz). To measure PKC α activation, phospho-specific antibody (Millipore) was used. The intensity of bands was quantified by densitometry using a Microcomputer Imaging Device version M5plus (Imaging Research). Alternatively, p38 activation was analysed with Flow Cytometric analysis using Alexa Fluor 488-conjugated P-p38 (T180/Y182) mouse mAb according to manufacturer's instructions (Cell Signaling Technology). The Cyflogic 1.1.1. (CyFlo, Turku, Finland) software was used for the analysis of the cytometry data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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