Syndecan-1 couples the insulin-like growth factor-1 receptor to inside-out integrin activation

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Summary

Syndecan-1 (Sdc1) engages and activates the αvβ3 (and/or αvβ5) integrin when clustered in human carcinoma and endothelial cells. Although the engagement is extracellular, the activation mechanism is cytoplasmic. This talin-dependent, inside-out signaling pathway is activated downstream of the insulin-like growth factor-1 receptor (IGF1R), whose kinase activity is triggered by Sdc1 clustering. In vitro binding assays using purified receptors suggest that association of the Sdc1 ectodomain with the integrin provides a 'docking face' for IGF1R. IGF1R docking and activation of the associated integrin is blocked by synstatin ($SSTN_{92–119}$), a peptide derived from the integrin engagement site in Sdc1. IGF1R colocalizes with αvβ3 integrin and Sdc1 in focal contacts, but fails to associate with or activate the integrin in cells either lacking Sdc1 or expressing $Sdc1^{\Delta67–121}$, a mutant that is unable to form the Sdc1–integrin–IGF1R ternary complex. Integrin activation is also blocked by IGF1R inhibitors or by silencing IGF1R or talin expression with small-interfering RNAs (siRNAs). In both cases, expression of the constitutively active talin F23 head domain rescues integrin activation. We recently reported that $SSTN_{92–119}$ blocks angiogenesis and impairs tumor growth in mice, therefore this Sdc1-mediated integrin regulatory mechanism might be a crucial regulator of disease processes known to rely on these integrins, including tumor cell metastasis and tumor-induced angiogenesis.

Key words: Syndecan, Synstatin, Heparan sulfate proteoglycan, Integrin, IGF1R

Introduction

The syndecans are multi-functional receptors expressed on the surface of all adherent cells. Sdc1 is prominently expressed on epithelial cells; its expression is altered in many cancers (Beauvais and Rapraeger, 2004) and is upregulated on activated endothelial cells (Elenius et al., 1991; Gallo et al., 1996; Kainulainen et al., 1996; Worapamorn et al., 2002). Similarly to other syndecan family members, it engages the extracellular matrix (ECM) via its heparan sulfate (HS) glycosaminoglycan chains that bind the 'heparin-binding' domains found in most ECM ligands, including vitronectin (VN) and fibronectin (FN), laminin, the fibrillar collagens and matricellular proteins (Alexopoulou et al., 2007; Bernfield et al., 1999; Woods, 2001). The syndecans share highly conserved cytoplasmic and transmembrane core protein domains, but it is their unique ectodomains that set the individual family members apart. Emerging evidence suggests that the syndecans can organize and regulate other cell surface receptors, including integrins, via this domain during matrix signaling (Beauvais and Rapraeger, 2004; Xian et al., 2009).

Sdc1 regulates the activation of αvβ3 and αvβ5 integrins on mammary carcinoma cells, fibroblasts and endothelial cells (Beauvais et al., 2004; Beauvais et al., 2009; Beauvais and Rapraeger, 2003; McQuade et al., 2006). Activation of αvβ3 and/or αvβ5 integrin stimulates adhesion, spreading and migration of tumor and endothelial cells, with clear consequences on tumor progression. Upregulated expression and activity of the αvβ3 integrin is a poor prognostic indicator in many cancers (Brooks et al., 1997; De et al., 2003; Felding-Habermann et al., 2002; Rolli et al., 2003), where the activated integrin facilitates tumor cell growth, survival and metastasis (Felding-Habermann et al., 2001; Liapis et al., 1996). During tumor-induced angiogenesis, endothelial cells upregulate both αvβ3 and αvβ5 integrin expression in response to tumor-released angiogenic factors such as vascular endothelial or fibroblast growth factors (Friedlander et al., 1995). Signaling by these integrins promotes endothelial cell proliferation, migration and survival, which are necessary for neovessel formation and tumor progression (Avraamides et al., 2008; Stupack and Cheresh, 2003). Thus, understanding the mechanism(s) by which Sdc1 regulates the activity of αvβ3 and αvβ5 integrins might lead to the development of new anti-cancer therapies.

Ligation and clustering of Sdc1, either on cells in suspension or bound to an immobilized ligand (Sdc1 antibody or VN), activates the αvβ3 and/or αvβ5 integrin, thus stimulating cell spreading and migration (Beauvais et al., 2004; Beauvais et al., 2009; Beauvais and Rapraeger, 2003; McQuade et al., 2006). Silencing of human Sdc1 expression by species-specific siRNAs blocks integrin activation, which can be rescued by ectopic expression of either full-length mouse Sdc1 or the glycosylphosphatidylinositol-anchored mouse Sdc1 ectodomain alone (GPI–S1ED) (Beauvais et al., 2004; McQuade et al., 2006). This traced the integrin activation site exclusively to the Sdc1 ectodomain and, using Sdc1 deletion mutants, to a 34 amino acid site lying between residues 88 and 121 (Beauvais et al., 2004). Via this site, Sdc1 associates directly with the αvβ3 and αvβ5 integrins (Beauvais et al., 2009). A 28-residue peptide derived from this site ($SSTN_{92–119}$) competes for the Sdc1–integrin interaction, blocks integrin activation and consequently, inhibits angiogenesis in vitro and in vivo and mammary carcinoma growth in nude mice (Beauvais et al., 2009).

Integrin activation, classically defined as a shift from a low affinity to a high affinity ligand-binding state, normally occurs in response to an energy-dependent, inside-out signal (Kassner et al., 1994; O'Toole et al., 1994). This shift results from conformational

changes in the integrin extracellular 'head' domains, causing them to extend and expose the ligand-binding site of the receptor. Since the integrin regulatory activity of Sdc1 appears to be confined to the Sdc1 ectodomain, this raised the question of whether Sdc1 serves purely a structural role (e.g. extracellular interaction of Sdc1 with the integrin supports and/or stabilizes shifts in the integrin extracellular domain), or whether ligation of Sdc1 causes an inside-out signal that activates the integrin.

Here, we report that Sdc1 activates the integrin via an inside-out signal. The ectodomain interaction of Sdc1 with αvβ3 or αvβ5 integrin provides a docking site that captures and activates the IGF1R, leading to autophosphorylation of IGF1R and activation of the integrin. This mechanism is independent of IGF1, but is enhanced by the growth factor. Importantly, whereas clustering of Sdc1 is sufficient to activate the IGF1R and thus the integrin, activation of IGF1R by clustering or by IGF1 stimulation fails to activate the integrin in the absence of Sdc1 or when engagement of the integrin by Sdc1 is blocked by $SSTN_{92–119}$. Thus, Sdc1 serves not only to activate the IGF1R, but also to functionally couple it to the integrin – coupling that is necessary for inside-out integrin activation.

Results

Sdc1-dependent activation of the αvβ3 integrin is energy dependent

Our previous work has shown that clustering of Sdc1 on either mammary carcinoma or endothelial cells leads to activation of the αvβ3 integrin (Beauvais et al., 2004; Beauvais et al., 2009), as shown by use of αvβ3-specific 'activation sensors', namely, the monovalent Fab fragment of the WOW1 ligand-mimetic monoclonal antibody (mAb) or the natural, multivalent ligand, fibrinogen (Fg) (Pampori et al., 1999). Integrin activation traditionally refers to energy-dependent, inside-out signaling that leads to a conformational shift in the integrin extracellular domain necessary for high-affinity ligand binding (Kassner et al., 1994; O'Toole et al., 1994). To question whether clustering of Sdc1 induces an energy-dependent signal, we tested the effects of metabolic inhibitors on Sdc1-induced integrin activation in MDA-MB-231 human mammary carcinoma cells. Integrin activation in these cells can be induced either by plating the cells on VN, which ligates the syndecan via its heparan sulfate chains, or an Sdc1-specific antibody substratum (Fig. 1) (Beauvais and Rapraeger, 2003). In these cells, integrin activation is facilitated by overexpressing Sdc1, which overrides trans-dominant inhibition by the α2β1 integrin (Beauvais and Rapraeger, 2003). For this reason, we used cells overexpressing full-length mouse Sdc1 and compared its effects with a mouse Sdc1 mutant that fails to engage the integrin ($Sdc1^{Δ67–121}$).

Stable mouse Sdc1 transfectants were treated with the metabolic inhibitors sodium azide (NaN_3) and/or 2-deoxyglucose (2-DOG) when plated on VN (Fig. 1A) or mouse Sdc1-specific mAb 281.2 (Fig. 1B). Treatment with either inhibitor alone blocks spreading on VN, and combined treatment shows that the effects are additive, because together they completely displace cells from the matrix ligand (Fig. 1A). Spreading is blocked with 1.0 μM $SSTN_{92–119}$, a peptide derived from the Sdc1 ectodomain that competitively blocks the formation of Sdc1–αvβ3-integrin complexes necessary for activation of the integrin (Beauvais et al., 2009), whereas an inactive SSTN peptide ($SSTN_{94–119}$) has no effect. The αvβ3-integrin-specific inhibitory mAb LM609 (30 μg/ml) also blocks their spreading. Although the concentration of LM609 was relatively high and might have nonspecific targets or effects, we have shown previously that it is the αvβ3 integrin on these cells that is responsible for this adhesion (Beauvais et al., 2004; Beauvais et al., 2009).

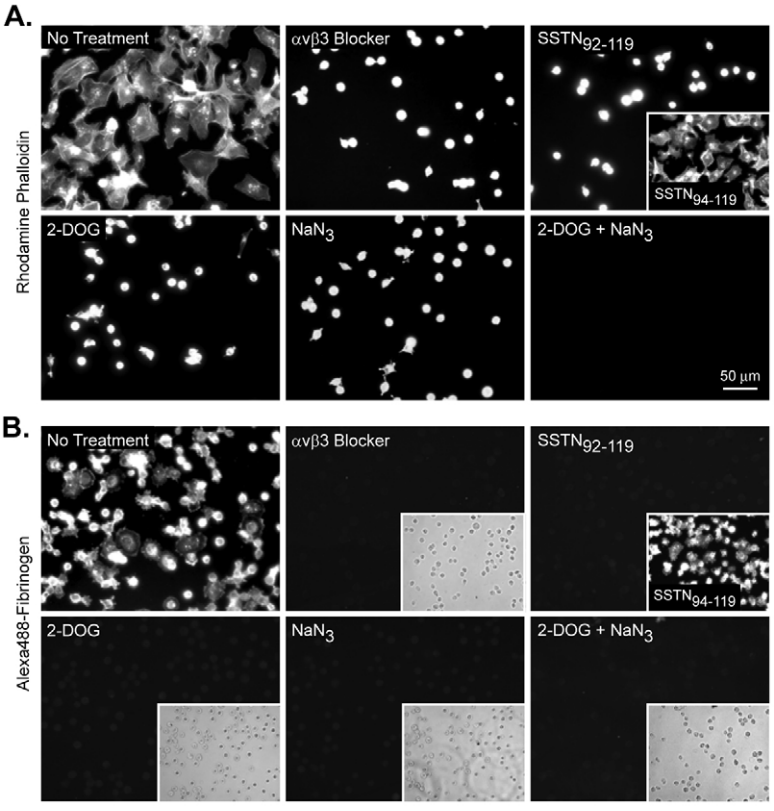

Fig. 1. Sdc1-dependent activation of αvβ3 integrin is energy dependent. MDA-MB-231 cells expressing mouse Sdc1 were plated on (**A**) VN (20 μg/ml) or (**B**) antibody-coated (10 μg/ml mAb 281.2) wells in plating medium alone or in medium containing 30 μg/ml mAb LM609, 1 μM SSTN, 50 mM 2-DOG, 0.07% NaN_3, or 50 mM 2-DOG plus 0.02% NaN_3. Cells were incubated at 37°C for 2 hours, fixed, permeabilized and stained with Rhodamine-conjugated phalloidin or Alexa-Fluor-488-conjugated Fg, respectively. Scale bar: 50 μm.

To determine whether the cells' response traces to a failure of syndecan-mediated integrin activation, we used a high-affinity fluorescent Fg probe on cells in which Sdc1 is engaged and clustered by a substratum of mAb 281.2 (Fig. 1B). In response to Sdc1 ligation, the cells spread and exhibit Fg binding, which indicates αvβ3 integrin activation (Pampori et al., 1999). However, treatment with 50 mM 2-DOG or 0.07% NaN_3 alone, or a combination of 50 mM 2-DOG plus 0.02% NaN_3 blocks cell spreading (insets, Fig. 1B) and also completely blocks Fg binding. As expected, the αvβ3 blocking antibody and $SSTN_{92–119}$ also block Fg binding.

Sdc1-dependent activation of the αvβ3 integrin requires activated IGF1R

These results suggest that Sdc1 engagement and clustering activates an inside-out signaling mechanism, perhaps by initiating either auto- or trans-phosphorylation of an associated kinase. To test this hypothesis, we screened a number of kinase inhibitors for their ability to block Sdc1-mediated activation of the integrin (data not shown). One set of inhibitors found to be active was a class of potent, cell-permeable IGF1R inhibitors, including tyrphostin AG538 and picropodophyllin (PPP). These are specific for this kinase because they target its activation loop and/or substrate binding site (Blum et al., 2000; Girnita et al., 2004; Vasilcanu et al., 2004). Inhibition of IGF1R autophosphorylation by these inhibitors is documented later (Fig. 6D).

Treatment of mouse Sdc1-expressing MDA-MB-231 cells with these IGF1R inhibitors completely blocks their spreading on VN (Fig. 2A,B) and mouse Sdc1 mAb 281.2 (Fig. 2C), indicating a lack of activated αvβ3 integrin, which was further confirmed by the failure of cells to bind soluble Fg (insets, Fig. 2C). This is specific for αvβ3 because these inhibitors had no effect on cell attachment and spreading on FN (Fig. 2A) or type-I collagen (supplementary material Fig. S1), which is α5β1 specific or α2β1 specific, respectively (Beauvais et al., 2004; Beauvais and Rapraeger, 2003), and, active αvβ3 integrin cannot be detected by fluorescent Fg staining of cells plated on these matrix ligands (supplementary material Fig. S1) (Beauvais et al., 2004; Beauvais and Rapraeger, 2003). Although IGF1R kinase activity appears to be required, this response does not require IGF1. Treatment with IGF1R antibody mAb 24-57, which blocks IGF1 binding (Schumacher et al., 1993; Soos et al., 1992), has no effect on the Sdc1-dependent mechanism (Fig. 2D), suggesting that ligation of Sdc1 directly activates the kinase. Nonetheless, treatment with exogenous IGF1 enhances activation of the αvβ3 integrin, as indicated by increased Fg binding and more prominent integrin clusters on the cells (Fig. 2D). This requires interaction of Sdc1with the integrin, because the mouse $Sdc1^{\Delta 67–121}$ mutant, which cannot interact with or activate the integrin (Beauvais et al., 2004; Beauvais et al., 2009), fails to induce spreading even in the presence of exogenous IGF1 (Fig. 2D).

We next examined whether perturbation of IGF1R affects αvβ3-integrin-dependent migration using a modified Boyden chamber assay. Indeed, treatment with either AG538 or PPP blocks MDA-MB-231 cell migration across VN-coated filters by over 60% in response to either 20 ng/ml EGF (Fig. 2E, black-filled bars) or 10% serum (data not shown). Similar effects were observed when IGF1R expression was silenced using siRNA (Fig. 2E). This IGF1R-dependent migration is also dependent on Sdc1, because it is blocked by $SSTN_{92–119}$ (Fig. 2E). By contrast, none of these treatments have any effect on the ability of the cells to migrate across FN (Fig. 2E, gray-filled bars).

Although inhibition or silencing of IGF1R blocks αvβ3-dependent migration, treatment with IGF1 enhances their response to EGF when migrating across VN, but not FN (Fig. 2E). We found that IGF1 alone also stimulates cell migration, which again is specific for VN (Fig. 2F). Cell migration is inhibited by treatment with mAb 24-57, which blocks IGF1R, as expected (Fig. 2F). The $SSTN_{92–119}$ peptide (Fig. 2F) also reduces migration to levels observed after IGF1R inhibitor or siRNA treatment. These findings confirm the apparent increase in integrin activation levels observed when cells are plated on Sdc1 antibody in the presence of IGF1 (cf. Fig. 2D) and suggest that Sdc1 has two roles: (1) to activate the IGF1R even in the absence of IGF1, and (2) based on the $Sdc1^{\Delta 67–121}$ and SSTN findings, to couple active IGF1R to an integrin activation mechanism.

Formation of an Sdc1–αvβ3-integrin–IGF1R ternary complex relies on the Sdc1 ectodomain

Because we have shown that the Sdc1 ectodomain interacts directly with the αvβ3 integrin (Beauvais et al., 2009), it is possible that this interaction serves to capture the IGF1R. This hypothesis was tested by co-immunoprecipitation studies. Sdc1 immunoprecipitates were isolated (Fig. 3A) using antibodies directed against either endogenous human (mAb B-A38) or ectopically expressed mouse Sdc1 constructs (mAb 281.2). Immunoblotting reveals IGF1R and β3 integrin (but not β1 integrin) in the immune complexes isolated from MDA-MB-231 cells expressing either empty vector (NEO) or full-length Sdc1 (Fig. 3A). By contrast, neither of these receptors associates with $Sdc1^{\Delta 67–121}$. Immunoprecipitates for the individual integrins (isolated from HUVEC whole-cell lysates) were used as comparative positive controls (Fig. 3B). In a parallel approach, we found that $SSTN_{92–119}$ efficiently disrupts association of the β3 integrin and IGF1R with both human Sdc1 and mouse Sdc1, but the $SSTN_{94–119}$ control peptide does not (Fig. 3A).

To demonstrate that $SSTN_{92–119}$ specifically competes for Sdc1 binding in the Sdc1–αvβ3–IGF1R complex, we used biotin label transfer (supplementary material Fig. S2). We have previously shown that biotinylated $SSTN_{92–119}$ binds and transfers its biotin label to purified integrin (Beauvais et al., 2009). Here, $SSTN_{92–119}$ (competitive peptide) and $SSTN_{94–119}$ (control, non-competitive peptide) were labeled with UV-photoactivatable biotin transfer reagent, and then incubated with human dermal microvascular endothelial cells (HMEC-1) in culture. Crosslinking and transfer of the biotin label under reducing conditions identified SSTN-interacting proteins at ~100, 110, 115, 130 and 135 kDa. Immunoblotting demonstrates that these correspond to IGF1Rβ subunit, the β3, β5 and αv integrin subunits and the IGF1Rα subunit, respectively.

The IGF1R has been reported to cooperate and associate with both the αvβ3 and αvβ5 integrins (Brooks et al., 1997; Clemmons and Maile, 2005; De et al., 2003; Mira et al., 1999; Schneller et al., 1997). In addition, we have shown that not only the αvβ3 integrin, but also the αvβ5 integrin binds directly to Sdc1 and is dependent on Sdc1 for its activation (Beauvais et al., 2009; McQuade et al., 2006). Thus, we next tested whether the association of IGF1R with these integrins depends on Sdc1. Purified IGF1R was immobilized on antibody-coated beads and used as 'bait' to capture purified integrin (either αvβ3 or αvβ5) added in solution as 'prey'. Surprisingly, we found that IGF1R alone fails to capture observable amounts of either integrin (Fig. 3B). However, IGF1R does capture both integrins if either of them is combined in solution with glutathione S-transferase (GST)-fused mouse Sdc1 ectodomain

(S1ED; Fig. 3B); this binding is blocked by $SSTN_{92–119}$, but not by the inactive $SSTN_{94–119}$ peptide. GST alone, used as a negative control, fails to bind either integrin in the presence of IGF1R. As an additional control, the α5β1 integrin also fails to bind GST–S1ED in the presence of IGF1R.

To test whether Sdc1, rather than the integrin, mediates the interaction with the IGF1R, mouse S1ED immobilized to glutathione beads was used as bait to capture purified IGF1R (Fig. 3C). Little if any binding was observed, but mouse S1ED does capture IGF1R in the presence of either the αvβ3 or αvβ5 integrin and is disrupted by $SSTN_{92–119}$. Together, these results suggest that Sdc1, the αvβ3 integrin (or αvβ5) and IGF1R form a ternary complex and that complex assembly requires that the syndecan and integrin engage each other via the SSTN site on the syndecan ectodomain.

Sdc1 expression drives IGF1R-dependent activation of the αvβ3 integrin necessary for endothelial cell migration

In another approach to show that Sdc1 is necessary for IGF1R association with αvβ3 integrin, we used HUVEC clones that are

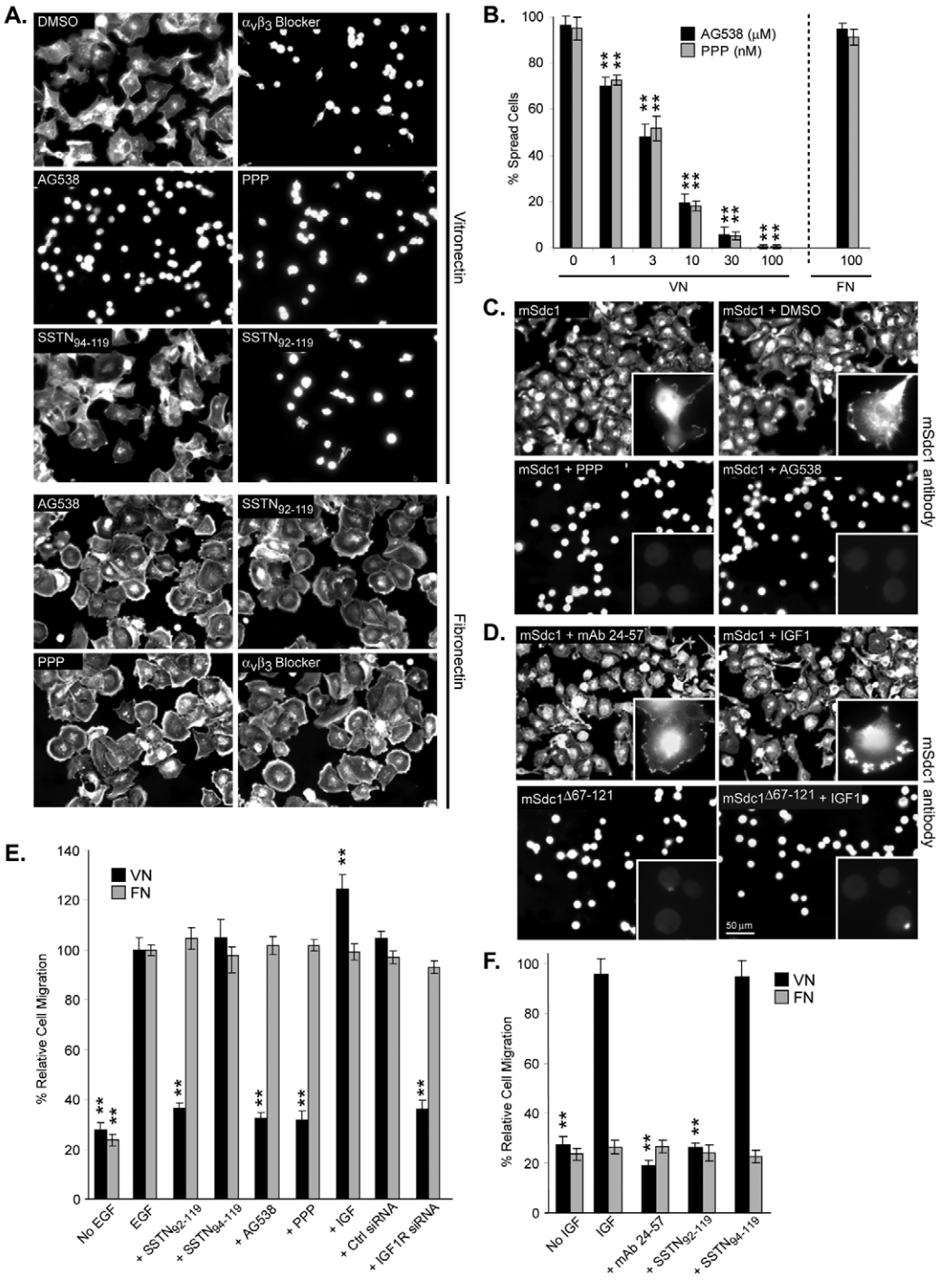

Fig. 2. Sdc1-dependent activation of the αvβ3 integrin requires activated IGF1R. MDA-MB-231 cells expressing mouse Sdc1 or mouse $Sdc1^{Δ67–121}$ were plated on (**A**,**B**) VN or FN-coated or (**C**,**D**) Sdc1 antibody-coated wells in plating medium containing vehicle alone (DMSO), 30 μg/ml mAb LM609, 1 μM SSTN, 10 μM AG538, 10 nM PPP, 1.5 μg/ml function-blocking IGF1R mAb 24-57 or 30 ng/ml IGF1. Cells were incubated at 37°C for 2 hours, fixed, permeabilized and stained with either Rhodamine-conjugated phalloidin or Alexa-Fluor-488-conjugated Fg (insets only). Scale bar: 50 μm (mean ± s.e.m.; $^{**}P<0.01$). (**E**,**F**) Non-siRNA transfected, control siRNA-transfected or *IGF1R* siRNA-transfected MDA-MB-231 cells were seeded on polycarbonate filters coated with either VN (black) or FN (gray) in a modified Boyden chamber. Cells were plated in plating medium alone or medium containing 1 μM SSTN, 10 μM AG538, 10 nM PPP, 1.5 μg/ml function-blocking IGF1R mAb 24-57 or 30 ng/ml IGF1. After 16 hours, cells that migrated through the filter in response to either EGF (E) or IGF1 (F) as a chemoattractant in the lower chamber were quantified by colorimetric staining (mean ± s.e.m.; $^{**}P<0.01$).

either positive [HUVEC(+)] or negative [HUVEC(–)] for Sdc1 expression (Figs 4 and 5). We have previously used the HUVEC(–) cells to demonstrate that integrin activation can occur in the absence of *SDC1* gene expression, but it occurs via a compensatory mechanism that is refractory to $SSTN_{92–119}$ treatment (Beauvais et al., 2009). The HUVEC clones display equivalent levels of IGF1R (Fig. 5A) and αvβ3 integrin expression (Beauvais et al., 2009) by flow cytometry.

Immunostaining adherent cells for Sdc1, the αvβ3 integrin and IGF1R (relative to matched IgG controls, supplementary material Fig. S3), we found that Sdc1 and the αvβ3 integrin (as well as the αvβ3 integrin and IGF1R), colocalize at dense, peripheral focal contacts or focal adhesions in the Sdc1-positive HUVECs, sites that also stain positively for phosphotyrosine (Fig. 4). Intriguingly, the Sdc1-negative HUVECs display a similar staining pattern for the αvβ3 integrin as their Sdc1-positive counterparts, but the IGF1R fails to colocalize with the integrin at these adhesion sites, again suggesting that the association of IGF1R with the integrin requires Sdc1. This was further confirmed by immunoprecipitation of the IGF1R from HUVEC(+) cells, which captures the αvβ3 and αvβ5 integrins, as well as Sdc1. By contrast, IGF1R fails to capture either the αvβ3 or αvβ5 integrin in HUVEC(–) cells (Fig. 5B).

Last, we examined the functional consequence of IGF1R association (or lack thereof) with the integrin using an 18 hour scratch wound assay. We found that both HUVEC(+) and (–) cells migrate to close the wound in response to stimulation with vascular endothelial growth factor (VEGF) (Fig. 5C,D). However, in the HUVEC(+) cells migration is severely blunted by treatment with $SSTN_{92–119}$ or the IGF1R inhibitor, PPP. By contrast, these inhibitors have no effect on HUVEC(–) cell migration; however, the migration of both cell lines is blocked by the αvβ3 integrin inhibitory mAb LM609 (Fig. 5C,D). These results suggest that in cells in which αvβ3 integrin activation depends on IGF1R, Sdc1 is required to couple IGF1R to the integrin and to activate the kinase.

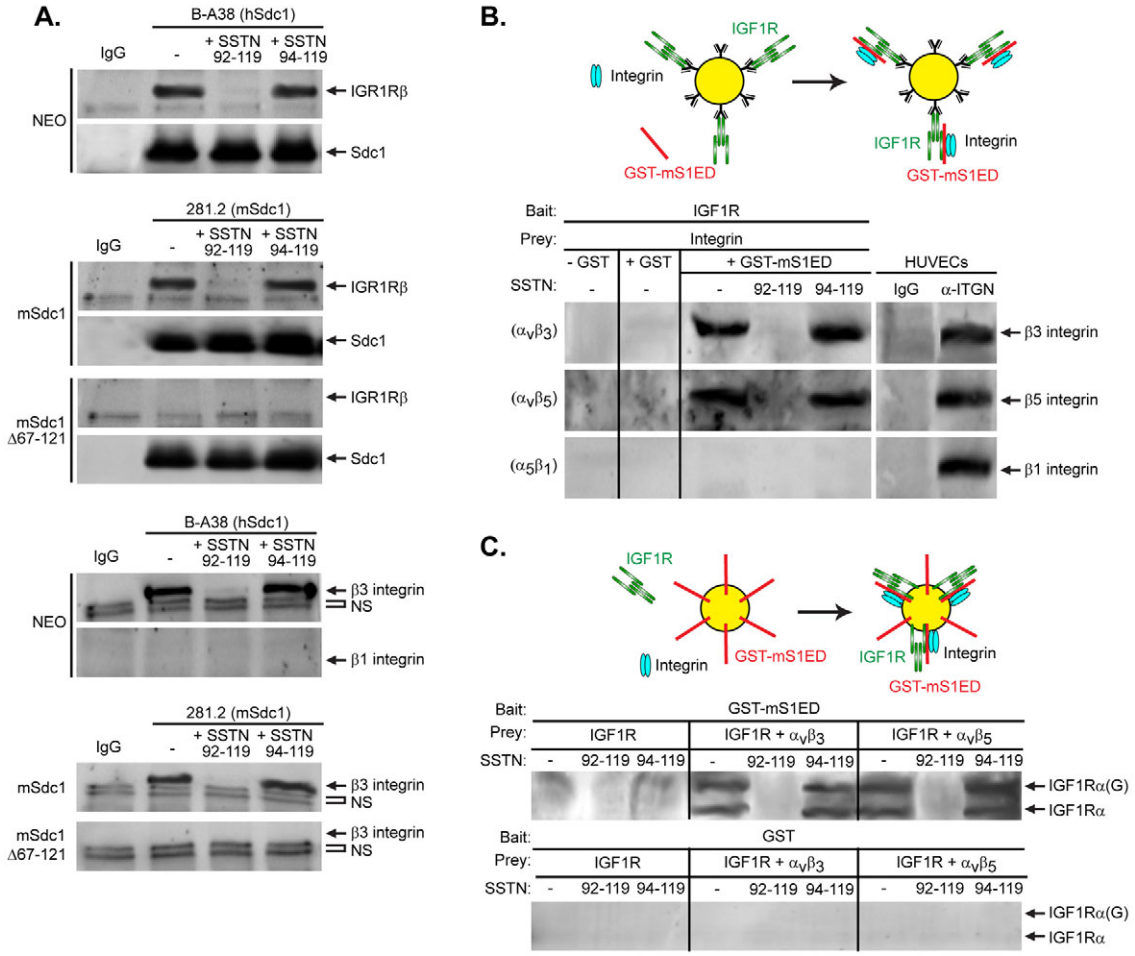

Fig. 3. Sdc1, IGF1R and the αvβ3 or αvβ5 integrin form a ternary complex that relies on the Sdc1 ectodomain. (**A**) Human Sdc1 and mouse Sdc1 were immunoprecipitated (using mAb B-A38 and 281.2, respectively) from MDA-MB-231 cells transfected with empty vector (NEO), mouse Sdc1 or mouse $Sdc1^{Δ67–121}$. Immunoprecipitations were conducted in the presence or absence of 1 μM SSTN peptide. Blots were probed for co-precipitation of the β3 (~105 kDa), β5 (~100 kDa) or β1 (~130 kDa) integrin subunit or the IGF1Rβ (~ 95 kDa) subunit with Sdc1. Note the nonspecific bands (NS) that appear in all lanes, including the IgG isotype control precipitations. (**B**) IGF1R immobilized to IGF1R antibody-coated beads was incubated with purified integrin alone or purified integrin plus GST–S1ED in the presence or absence of SSTN peptide. As a comparative control, individual integrins were immunoprecipitated from HUVEC whole-cell lysates using mouse mAbs 23C6, 15F11 and HA5 (4 μg/ml) against human αvβ3, αvβ5, α5β1 integrin, respectively. Captured integrin was detected on blots by probing for the integrin β-subunits. (**C**) Glutathione beads bearing GST alone or GST–S1ED were incubated with purified integrin alone or purified integrin plus IGF1R in the presence or absence of SSTN peptide. Captured IGF1R was detected on blots by probing for the IGF1Rα subunit, which exists in a non-glycosylated (IGF1Rα; ~130 kDa) or glycosylated form [IGF1Rα(G); ~150 kDa].

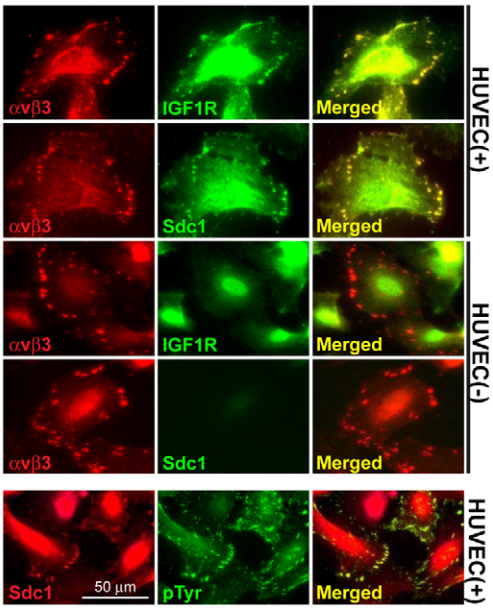

Fig. 4. Localization of IGF1R at the αvβ3 integrin adhesion sites requires Sdc1. Sdc1-positive and negative HUVECs were co-stained for αvβ3 integrin (mouse mAb LM609) and IGF1R (chicken anti-IGF1Rα), αvβ3 integrin (mouse mAb LM609) and Sdc1 (rabbit anti-human-S1ED) or Sdc1 (rabbit anti-human-S1ED) and anti-phosphotyrosine (mouse mAb PY20) followed by Alexa-Fluor-488 (green)- and Alexa-Fluor-546 (red)-conjugated secondary antibodies. Scale bar: 50 μm.

Clustering of Sdc1 activates IGF1R leading to αvβ3 integrin activation

The model that emerges from these data is that clustering of Sdc1 upon matrix engagement causes co-clustering and autophosphorylation of the IGF1R. We have shown previously by positive WOW1 binding that clustering of Sdc1 on suspended cells induces activation of the αvβ3 integrin (Beauvais et al., 2004; Beauvais et al., 2009). Using the same approach here, we found that antibody-induced clustering of mouse or human Sdc1 on suspended MDA-MB-231 or HUVEC(+) cells, respectively, activates the αvβ3 integrin (measured by FACS analysis of WOW1 binding), and this is blocked upon silencing IGF1R expression by ~95% (Fig. 6A,B). Integrin activation in response to Sdc1 clustering [ectopic mouse Sdc1 on MDA-MB-231 or endogenous human Sdc1 on HUVEC(+) cells] is also blocked by treatment with IGF1R inhibitors, AG538 or PPP, and by competitive inhibition with $SSTN_{92–119}$ (Fig. 6C). By contrast, integrin activation is enhanced by the addition of exogenous IGF1 (Fig. 6C). Therefore, although IGF1 is not required for Sdc1-mediated activation of the integrin (i.e. clustering of the syndecan alone is sufficient), it nonetheless can enhance it (cf. Fig. 2D). Importantly, clustering of mouse $Sdc1^{\Delta67–121}$, which lacks the crucial integrin–IGF1R interaction site, fails to activate the integrin (Fig. 6C), and this could not be rescued by IGF1.

We next examined activation of the IGF1R itself by monitoring phosphorylation of Y1131 in the activation loop of the kinase (Fig. 6D). MDA-MB-231 cells expressing mouse Sdc1 or $Sdc1^{\Delta67–121}$ and HUVEC(+) cells were serum-starved for 24 hours, suspended, treated with primary and secondary antibodies to cluster Sdc1 and then stained with a phospho-specific Y1131 IGF1R antibody (mAb K74-218). FACS analysis shows that levels of phosphorylated IGF1R increase fivefold in response to Sdc1 clustering; clustering of the $Sdc1^{\Delta67–121}$ mutant does not induce phosphorylation of the receptor (Fig. 6D). Importantly, treatment with either $SSTN_{92–119}$ or IGF1R inhibitor blocks IGF1R phosphorylation (Fig. 6D), suggesting that the IGF1R undergoes autophosphorylation in response to clustering because of its interaction with Sdc1. Similarly to activation of the integrin (Fig. 6C), IGF1 enhances IGF1R phosphorylation when Sdc1 is clustered. However, unlike activation of the integrin, IGF1 does rescue IGF1R phosphorylation in the presence of clustered mouse $Sdc1^{\Delta67–121}$ (Fig. 6D). These data suggest that the Sdc1 integrin-binding site couples the integrin to the activated IGF1R; the mutant lacks this site and cannot assemble the complex. Thus, the integrin is not activated despite the presence of activated IGF1R.

To test the role of Sdc1 in coupling activated IGF1R to the process of integrin activation, IGF1R was activated directly, either by clustering the receptor using function-activating IGF1R mAb 3B7, or by adding exogenous IGF1. These two approaches yielded activation levels comparable with that seen with clustered Sdc1, although treatment with IGF1 was consistently higher (Fig. 6E); both treatments induce integrin activation as measured by WOW1 binding. However, treatment with $SSTN_{92–119}$ uncouples activation of the integrin from that of the IGF1R. These results confirm our postulated roles for Sdc1 in the process of αvβ3 integrin activation, namely that activation of the IGF1R upon matrix engagement and coupling the activated IGF1R to the integrin are prerequisite steps to integrin activation.

Sdc1–IGF1R-mediated activation of the αvβ3 integrin requires talin

The most proximal steps in inside-out integrin activation are talin activation and its binding to the integrin β-subunit cytoplasmic tail (Calderwood et al., 1999; Tadokoro et al., 2003; Vinogradova et al., 2002). Talin binding (via its head domain) disrupts a salt bridge between the α- and β-subunits, stabilizes the helical structure of the β-subunit cytoplasmic membrane-proximal region, and reorients the transmembrane β-helix leading to separation of the integrin tails (Kim et al., 2009; Lau et al., 2009; Wegener et al., 2007). This translates to the extracellular ‘head’ domains of the integrin subunits, which separate and extend leading to activation of the integrin (Anthis et al., 2009; Takagi et al., 2002; Xiong et al., 2001).

To establish whether talin has a role in Sdc1–IGF1R-mediated activation of the αvβ3 integrin, we used RNA interference to knock down talin expression in MDA-MB-231 cells expressing mouse full-length Sdc1. Introduction of siRNA to knock down human-specific talin (directed against the C-terminal rod domain) in these cells greatly reduces talin expression in a concentration-dependent manner relative to a control siRNA (Fig. 7A), but does not affect αvβ3 integrin expression, which was monitored by immunoblotting for the β3 subunit (Fig. 7A) and by cell surface FACS analysis using mAb LM609 (data not shown). As expected, downregulation of talin decreases αvβ3 integrin activity, as indicated by the inability of cells to spread on mouse-Sdc1-specific antibody mAb 281.2 (Fig. 7C). Expression of the wild-type mouse talin head domain (WT F23) in these cells (not targeted by the human-specific siRNA) rescues αvβ3 integrin activity (Fig. 7B) because the cells regain their ability to spread on Sdc1 antibody and also bind Fg with high affinity (Fig. 7C). WT F23 also rescues integrin activity in cells where IGF1R activity is blocked by PPP treatment (Fig. 7C). As a control, siRNA-transfected and PPP-

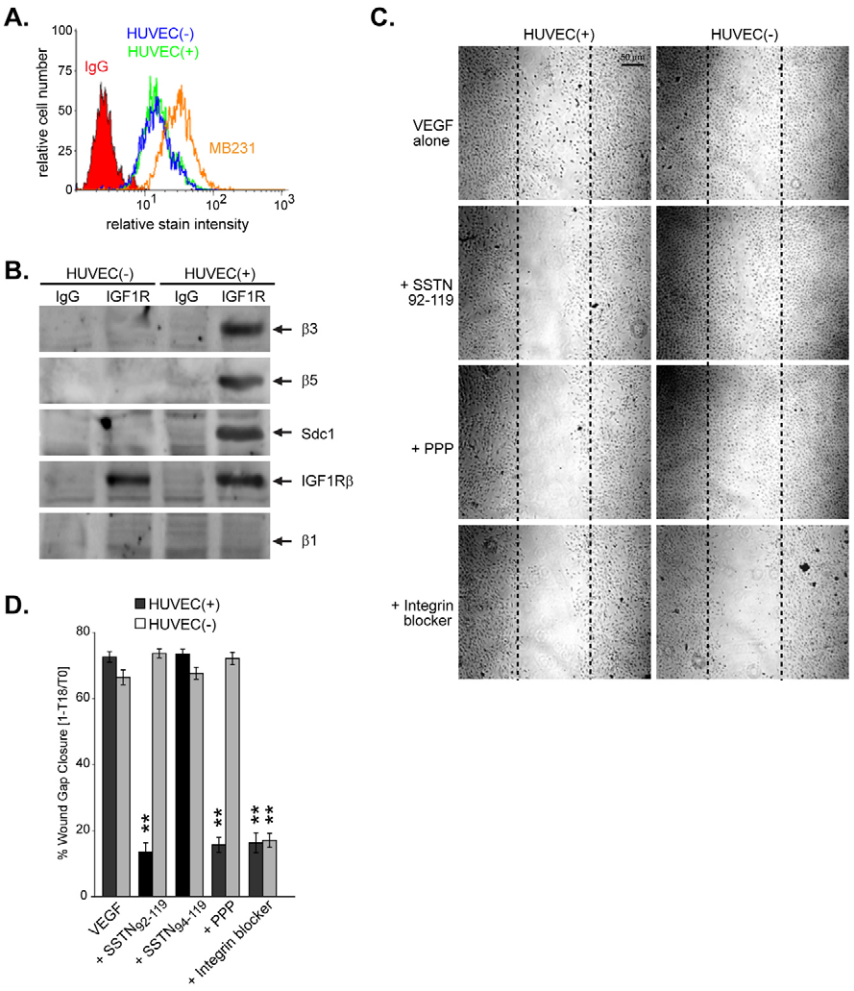

Fig. 5. Sdc1 expression drives IGF1R-dependent activation of the αvβ3 integrin necessary for endothelial cell migration. (**A**) FACS analysis of IGF1R expression in MDA-MB-231 human mammary carcinoma cells and HUVECs against an IgG isotype control. (**B**) IGF1R was immunoprecipitated from HUVEC clones (grown in serum-containing medium) positive or negative for Sdc1 expression. Blots were probed for co-precipitation of the β3 (~105 kDa), β5 (~100 kDa) or β1 (~130 kDa) integrin subunit or human Sdc1 (~85 kDa). (**C**,**D**) Confluent monolayers of Sdc1-positive or negative HUVECs were serum starved before wounding and then washed twice in SFM to remove suspended cells. The cells were further cultured in SFM containing VEGF alone or VEGF plus 1 μM SSTN, 10 nM PPP or 30 μg/ml mAb LM609. The wound site was photographed immediately after wounding (T_0) and again 18 hours later. Mean percentage wound closure (± s.e.m.) was calculated using the equation $[1-(T_{18}/T_0)]\times100$. (**$P$<0.01).

treated cells were also transfected with W359A F23 (Fig. 7B), a talin head domain point mutant that is unable to bind the integrin β-tail and thus cannot activate the integrin (Tadokoro et al., 2003; Wegener et al., 2007). In the presence of this mutant, the integrin remains inactive; the cells fail to spread in response to Sdc1 ligation and fail to bind Fg (Fig. 7C). These results suggest that talin is activated downstream of the IGF1R and has a role in Sdc1–IGF1R-mediated activation of the αvβ3 integrin.

Discussion

The current study extends our previous work and demonstrates that Sdc1 regulates activation of the αvβ3 and αvβ5 integrins by physically coupling these integrins to the IGF1R in human mammary carcinoma and endothelial cells. Sdc1 clustering in response to cells engaging either VN or Sdc1-specific antibody leads to IGF1R autophosphorylation. This in turn initiates an energy-dependent, inside-out signaling mechanism that activates the αvβ3 and/or αvβ5 integrin. Inside-out integrin activation (affinity modulation) is thought to occur via the binding of cytoplasmic proteins to the integrin β-subunit tail (Legate and Fassler, 2009; Legate et al., 2009; Liu et al., 2000) – most notably talin (Tadokoro et al., 2003; Wegener et al., 2007) – resulting in the sequential breakage of a cytoplasmic α-subunit–β-subunit salt bridge (Hughes et al., 1996), separation of the integrin transmembrane domains (Hoefling et al., 2009; Moser et al., 2009; Wegener and Campbell, 2008) and structural rearrangements in the extracellular integrin domains, resulting in high-affinity ligand binding (Arnaout et al., 2005; Askari et al., 2009; Luo et al., 2007; Xiong et al., 2009). Other proteins have a role in this process, including β3-endonexin (Kashiwagi et al., 1997; Shattil et al., 1995), kindlins (Larjava et al., 2008; Plow et al., 2009), migfilin (Ithychanda et al., 2009) and/or members of the Mig-10/RIAM/Lamellipodin protein family (Banno and Ginsberg, 2008; Han et al., 2006; Lee et al., 2009; Watanabe et al., 2008). At this juncture, it appears that talin is the ultimate target of the IGF1R mechanism; work to elucidate the signal transduction pathway by which IGF1R activates talin is ongoing.

Although our data clearly support a role for Sdc1 in inside-out activation of the integrin, they do not exclude the possibility that Sdc1 also participates in integrin avidity changes due to its direct interaction with the integrins. Integrin avidity (clustering) refers to changes in integrin diffusion, including homo-oligomerization of the integrin subunits (Li et al., 2004; Li et al., 2005b) and/or extracellular interactions of the integrin with other membrane proteins (Brown, 2002; Hemler, 2001; Humphries et al., 2004; Porter and Hogg, 1998). Changes in integrin avidity are not mutually exclusive from changes in affinity. Indeed, they complement or enhance inside-out affinity changes (Yauch et al., 1997) and appear to be crucial for linking the ligated integrin to the actin cytoskeleton during outside-in signalling, which is necessary for focal adhesion formation and substrate traction leading to cell migration (Hato et al., 1998; Li et al., 2005a). The

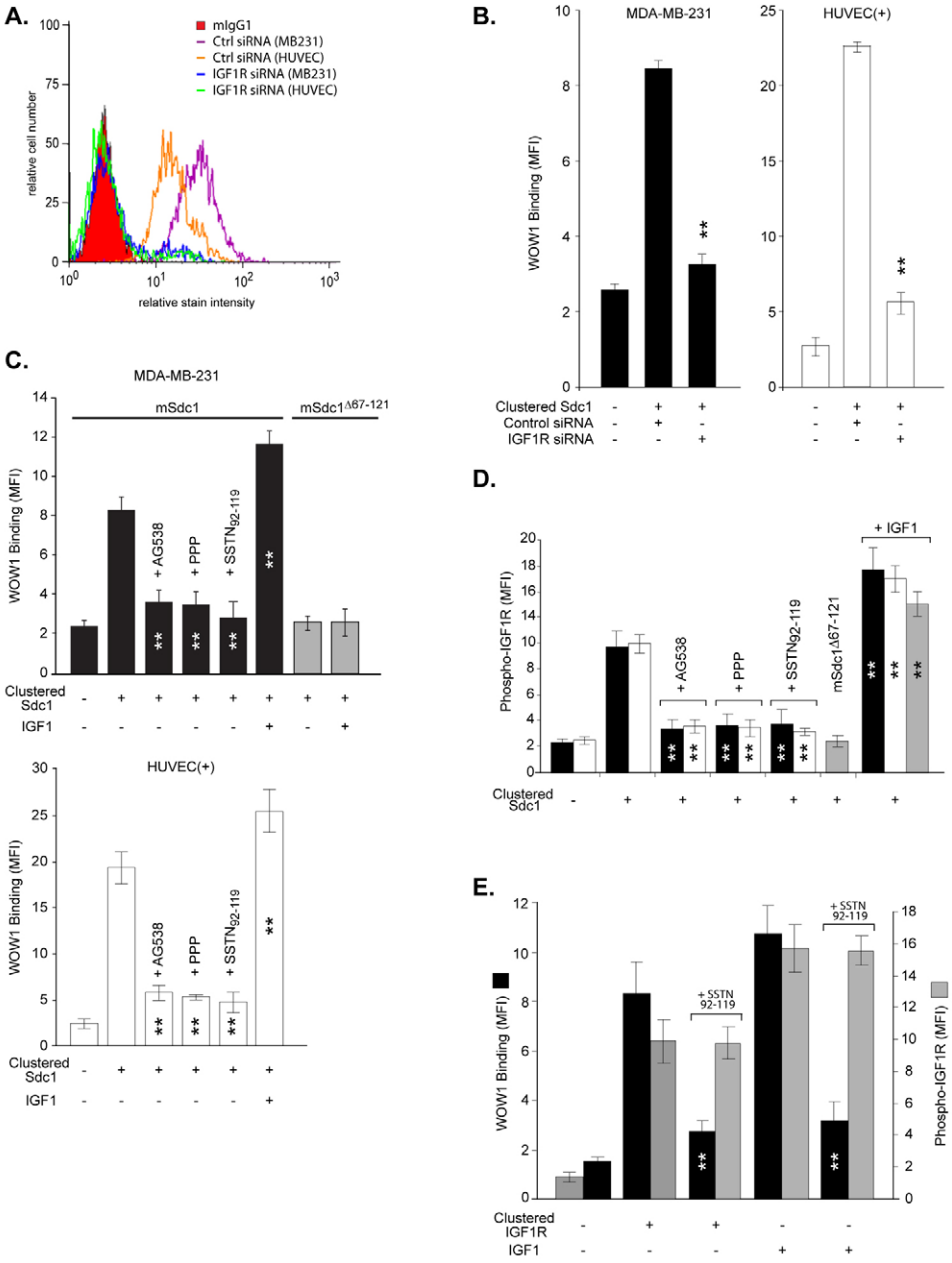

Fig. 6. Clustering of Sdc1 activates IGF1R leading to αvβ3 integrin activation. (**A**) FACS analysis for IGF1R expression in control or *IGF1R* siRNA-transfected MDA-MB-231 cells expressing mouse Sdc1 and HUVEC(+) cells. (**B–D**) Suspended cells in which mouse Sdc1 (black bars), mouse Sdc1$^{\Delta 67-121}$ (gray bars) or human Sdc1 (open bars) or (**E**) IGF1R was or was not clustered (in the presence or absence of IGF1) were fixed and labeled with either WOW1 mouse Fab followed by an Alexa-Fluor-488-conjugated secondary (B,C,E) or an Alexa-Fluor-647-conjugated phospho-specific IGF1R mAb K74-218 (D,E) and analyzed by FACS. The MFI for levels of activated αvβ3 integrin (WOW1) and phosphorylated IGF1R are depicted (mean ± s.e.m.; $^{**}P<0.01$).

fact that Sdc1 colocalizes with the integrin and IGF1R at these adhesion sites might indicate that Sdc1 acts both upstream (inside-out) and downstream (outside-in) of integrin activation to facilitate stable integrin signaling. In the syndecan family, Sdc4 is a well-described component and regulator of focal adhesions, particularly on FN matrices (Morgan et al., 2007; Woods and Couchman, 2001), whereas reports of Sdc1 and other syndecans at these sites are infrequent (Ishiguro et al., 2000; Yamagata et al., 1993). Whether the Sdc1-enriched sites are transient focal contacts or stable focal adhesions is not clear; their identity and the role of Sdc1 in these structures will be the subject of future study.

A clear role for Sdc1 at these adhesion sites is localization of IGF1R to the integrin and activation of an inside-out signaling pathway. We envision that the IGF1R docking site involves individual binding regions on both the syndecan and the integrin. However, these individual interactions are sufficiently low in affinity that we can neither recapitulate them in vitro using purified components (Fig. 3), nor can we immunoprecipitate the IGF1R and integrin together from cells that lack Sdc1 expression. However, a clear interaction is observed when both Sdc1 and the integrin are engaged and provided to the IGF1R. These data suggest a model whereby an assembled syndecan–integrin protein complex provides a binding face to which the IGF1R docks with higher affinity. An alternative hypothesis is that the interaction of integrin with the syndecan alters or locks the integrin ectodomain (or vice-versa) into a conformation that highly favors IGF1R binding.

By facilitating docking of the IGF1R to the Sdc1–integrin complex, Sdc1 accomplishes two major roles. First, it induces activation of the IGF1R when the complex is clustered in response to ECM engagement. This is shown most clearly by clustering the complex artificially using Sdc1-specific antibodies (Fig. 6). The IGF1R undergoes phosphorylation on Y1131 within its kinase domain, and this phosphorylation is blocked by IGF1R-specific inhibitors – strongly suggesting that autophosphorylation occurs

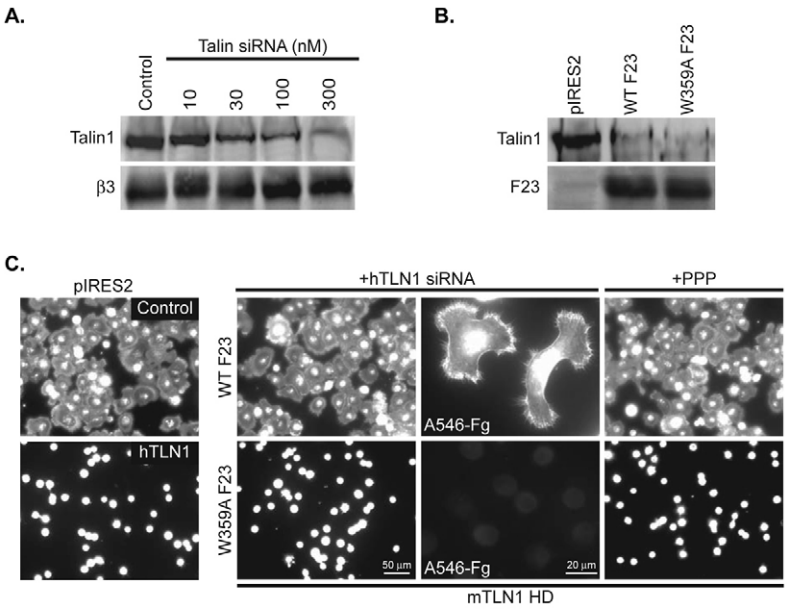

Fig. 7. Sdc1–IGF1R-mediated activation of the αvβ3 integrin requires talin. (**A**) Western blot of lysates collected from MDA-MB-231 mouse Sdc1-expressing cells transfected with control siRNA or increasing doses of human *TLN1*-specific siRNA and probed for expression of talin (~225 kDa; mAb TA205) or β3 integrin (~105 kDa; Fire and Ice) (**B**) Western blot for expression of full-length talin (mAb TA205) and HA-tagged talin head domain constructs, WT or W359A F23 (~25 kDa; anti-HA mAb 12CA5) in MDA-MB-231 mouse Sdc1-expressing cells co-transfected with human *TLN1* siRNA or control siRNA (200 nM) and talin head domain or empty vector (pIRES2). (**C**) siRNA- and 10 nM PPP-treated cells transfected with empty vector (pIRES2) or talin head domain (WT or W359A F23) spreading on mouse Sdc1-specific mAb 281.2. Cells visualized by expression of EGFP or by staining with Alexa-Fluor-546-conjugated Fg (A546–Fg) to specifically stain activated αvβ3 integrin.

between clustered IGF1Rs. This is unusual for an insulin receptor family member, because activation traditionally occurs by shifting the proximity of the two kinase domains within a single receptor in response to ligand binding, rather than by clustering to form receptor dimers or multimers. Nonetheless, it has been shown that clustering of the IGF1R induces its activation (Cara et al., 1988; Ikari et al., 1988; Takahashi et al., 1995; Xiong et al., 1992; Xu et al., 1991) – a technique that we recapitulated in our studies using IGF1R-specific antibody, which induced IGF1R activation (phosphorylation of the receptor kinase activation loop) at levels similar to that obtained by clustering Sdc1 (Fig. 6E).

The second major role of complex formation is that IGF1R–integrin docking appears to be a requirement for activation of the integrin. That is, activation of the IGF1R by artificial antibody clustering, or by addition of IGF1, fails to activate the integrin if the kinase is not part of the Sdc1–integrin complex. This is observed when: (1) Sdc1 expression is silenced [either by siRNA transfection or stochastically, as in the HUVEC(–) cells], (2) the $Sdc1^{\Delta 67–121}$ mutant that is unable to engage the integrin is expressed, or (3) the SSTN peptide that competitively displaces Sdc1, and thus IGF1R, from the integrin is present. Similarly, IGF1-mediated activation of the IGF1R can supplement integrin activation (Fig. 6C,E), but only if the IGF1R is part of the receptor complex. Indeed, IGFs are chemoattractants for many cancer cells, probably because of the ability of the IGF1R to affect αvβ3 and αvβ5 integrin activation via the Sdc1-mediated mechanism described here (Fig. 2F).

It seems apparent that a protein essential for integrin activation is present within or recruited to the ternary receptor complex and is targeted by the IGF1R. Work to identify the unknown target(s) is ongoing and is crucial to fully understand the integrin activation mechanism. This target might be a direct substrate of the IGF1R kinase, such as the insulin receptor substrate-1 (IRS-1) or Src homology and collagen homology (SHC). These proteins bind to the IGF1R NPEY juxtamembrane motif via their phosphotyrosine-binding (PTB) domains (Craparo et al., 1995; Gustafson et al., 1995) and can also bind the integrin β3-tail (Clemmons et al., 2007; Vuori and Ruoslahti, 1994), further linking IGF1R directly to the integrin. Intriguingly, the integrin β-subunit bears a similar NPxY motif required for talin binding (via its PTB domain), and this interaction is regulated by a phosphotyrosine switch (Critchley, 2009; Oxley et al., 2008). Conceivably, if talin did bind to the IGF1R NPEY motif, its displacement as a consequence of IGF1R phosphorylation would free it to bind and activate the integrin. However, it is equally likely that the IGF1R simply phosphorylates talin directly to activate it (Critchley, 2009) or indirectly activates talin downstream of Rap1 GTPase activation (Banno and Ginsberg, 2008) via the CT10-related kinase (CRK)/CRK-SH3-domain-binding guanine nucleotide-exchange factor (C3G) complex (Bos et al., 2001; Kinbara et al., 2003). Whatever the mechanism, talin is clearly an IGF1R target. Loss of talin or inhibition of IGF1R activity both block Sdc1-mediated activation of the integrin, but integrin activity is rescued if the cells are given back WT F23 mouse talin head domain (Fig. 7C).

Although crosstalk between IGF1R and αvβ3 and αvβ5 integrin signaling has been previously documented, a central role for Sdc1 in this mechanism has not been appreciated until now. These two receptors crossregulate each other in a number of cell types, including cancer, endothelial and vascular smooth muscle cells (Bahr and Groner, 2005; Clemmons, 2007; Delafontaine et al., 2004). This crossregulation involves not only association of the integrin with IGF1R (Brooks et al., 1997; Clemmons and Maile, 2005; De et al., 2003; Mira et al., 1999; Schneller et al., 1997), but also integrin-mediated control over the localization and/or activation of IGF1R signaling components or regulators, including IRS-1, SHC and SH2-domain-containing proteins, tyrosine phosphatase-2 (SHP-2) and substrate-1 (SHPS-1) (Clemmons, 2007; Clemmons and Maile, 2005; Clemmons et al., 2007; Lee and Streuli, 1999; Vuori and Ruoslahti, 1994). Indeed, IGF1R stimulation enhances the ligand-binding affinity of the αvβ3 integrin, with no change in receptor expression levels (Jones et al., 1996; Maile et al., 2002), and αvβ3 stimulation enhances IGF1-mediated migration and proliferation (Maile et al., 2006a; Maile et al., 2006b; Xi et al., 2008). Moreover, integrin inhibition blocks IGF-stimulated migration (Clemmons et al., 1999; Doerr and Jones, 1996; Kabir-Salmani et al., 2003) and expression of a dominant-negative IGF1R construct inhibits MDA-MB-231 and MDA-MB-435 breast cancer cell invasion and metastasis in vitro and in vivo (Dunn et al., 1998; Sachdev et al., 2004). This correlates well with spontaneous

metastasis of several tumor types, which requires both integrin and IGF1R activity (Brooks et al., 1997). Ostensibly this is true not only in the invading tumor cells, but also in activated endothelial cells during tumor-induced angiogenesis (Bae et al., 1998; Han et al., 2003; Kim et al., 1998; Kondo et al., 2003; Lee et al., 2000; Shigematsu et al., 1999). These findings take on a new light given our current finding that Sdc1 is likely to be a regulator of these interactions, which can be inhibited with the SSTN peptide.

Materials and Methods

Materials

Matrix ligands include FN (provided courtesy of Donna Peters, University of Wisconsin, Madison, WI) and VN, purified from human plasma as described in (Yatohgo et al., 1988). Sdc1-specific antibodies include mouse anti-human-Sdc1 mAb B-A38 (Serotec, Raleigh, NC) and rat anti-mouse-Sdc1 mAb 281.2 (Jalkanen et al., 1985). Rabbit polyclonal antibodies against recombinant human S1ED were affinity-purified as previously described (Beauvais et al., 2004) and used for immunofluorescence. Mouse mAb F69-3G10 (courtesy of Guido David, University of Leuven, Belgium) was used for simultaneous detection of mouse and human Sdc1 on western blots after digestion with heparin lyases (David et al., 1992). Anti-αvβ3-integrin antibodies included mouse mAb LM609 (courtesy of David Cheresh, University California, San Diego and The Scripps Research Institute, CA) and rabbit polyclonal 'Fire and Ice' (courtesy of Peter Newman, Blood Research Institute, Blood Center of Southeastern Wisconsin, Kenosha, WI). Mouse mAbs 23C6, 15F11 and HA5 (Millipore, Billerica, MA) against human αvβ3, αvβ5, α5β1 integrin, respectively, were used for immunoprecipitation. Mouse anti-β1 and anti-αv-integrin mAbs N29 (Millipore) and 3F12 (courtesy of Scott Blystone, SUNY Upstate Medical University, Syracuse, NY), respectively and rabbit polyclonal antibodies against β5 and αv integrins (Abcam, Cambridge, MA) were used for western blotting. The ligand-mimetic Fab WOW1 (courtesy of Sanford Shattil, University California, San Diego) and Alexa-Fluor-488- or Alexa-Fluor-546-conjugated Fg (Invitrogen, Carlsbad, CA) were used to detect activated αvβ3 integrin (Pampori et al., 1999). IGF1R antibodies include function-blocking mouse mAb 24-57 (Millipore, Billerica, MA) or 1H7 (Serotec), function-activating mouse mAb 3B7 (BD Biosciences, San Diego, CA), Millipore mouse mAbs JBW902 (used for immunoprecipitations), JY202, 1-2 and 7G11 (used for western blotting), rabbit polyclonal antibody (CBL257, used for western blotting), chicken anti-IGF1Rα (06-429, used for immunofluorescence) and BD Biosciences mouse mAb K74-218 (used for FACS). Mouse anti-phosphotyrosine (mAb PY20, BD Biosciences) was used for immunofluorescence. Anti-talin head domain mouse mAb TA205 (Millipore) and rod domain mouse mAb 8d4 (Sigma) against wild-type human talin and anti-hemagglutinin (HA) mouse mAb 12CA5 (Roche Molecular Biochemicals, Indianapolis, IN) against HA-tagged mouse talin head F23 constructs were used for western blotting.

The SSTN peptides (80–90% pure), derived from the mouse Sdc1 ectodomain sequence, were purchased from GenScript Corporation (Scotch Plains, NJ) and the UW Biotechnology Center Peptide Synthesis Facility. Recombinant GST and GST–mouse-S1ED protein was prepared as previously described (Beauvais et al., 2004). Human IGF1R, αvβ3, αvβ5 and α5β1 integrin purified from placenta (greater than 95% pure) were purchased from US Biologicals (Swampscott, MA) and human recombinant VEGF-A165 and IGF1 were purchased from Peprotech (Rocky Hill, NJ). IGF1R (AG538 and PPP) and anaerobic glycolysis (2-deoxy-D-glucose) inhibitors were purchased from EMD Chemicals (La Jolla, CA). Sodium azide, an inhibitor of oxidative phosphorylation, was purchased from Sigma (St Louis, MO).

Cell culture

All cell lines were cultured as previously described (Beauvais et al., 2009). Culture medium (DME or MCDB131) contained 5.56 mM glucose.

Immunofluorescence

Approximately 3.5×10^5 cells were plated onto acid-etched glass coverslips in complete culture medium. After 2 days, the cells were washed in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), fixed for 20 minutes in 4% EM-grade paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) at 4°C followed by cold acetone at –20°C for 5 minutes. Cells were then rinsed in CMF-PBS, quenched with 0.1 M glycine, permeabilized with CMF-PBS containing 0.5% Triton X-100 for 5 minutes at room temperature (RT) and then blocked with blocking buffer (CMF-PBS containing 3% FBS) at 4°C overnight. Cells were stained with primary antibodies (10 μg/ml) diluted in blocking buffer for 1–2 hours at RT, rinsed in CMF-PBS, incubated for 1 hour with secondary antibody (1:500–1:1000 dilution) in blocking buffer, followed by washing in CMF-PBS and ddH_2O and then mounted to slides with Immunomount (Shandon Lipshaw, Pittsburgh, PA) containing 0.1 M n-propyl gallate (Sigma) added as an anti-fading agent. Images were acquired using a PlanApo 63× (1.4 NA) objective on a Microphot-FX microscope (Nikon, Garden City, NY) equipped with an Image-Point cooled CCD camera system into IMAGE-PRO PLUS software (Media Cybernetics, Bethesda, MD). Images were processed and colorized using Adobe Photoshop (Adobe Systems, San Jose, CA).

Cell attachment and spreading assay

Cell attachment and spreading assays on Sdc1-specific antibody, VN or FN and siRNA transfections were conducted as described previously (Beauvais et al., 2004; McQuade et al., 2006). IGF1R (GenBank™ accession number NM_000875.3) Validated siRNA ID 110754 [nucleotide annotation: ^{2699}GGAATACAGGAAGTATGGA(tt)^{2717}], Talin-1 (GenBank™ accession number NM_0062892.2) Silencer Select siRNA ID s14186 [nucleotide annotation: ^{6891}GCAGTTGACAGGACATTCA(tt)^{6909}] and *Silencer* control siRNAs (used for control transfections) were purchased from Applera Corporation (Foster City, CA). For *TLN1* siRNA experiments, cells were co-transfected with 3–10 μg (per $0.5–1.0\times10^6$ cells) of empty vector alone (pIRES2-EGFP, Clontech Laboratories, Mountain View, CA), HA-tagged WT F23 or W359A mouse Talin-1 head domain (originally provided courtesy of Mark Ginsberg, University California, San Diego, subcloned into pIRES-EGFP, and authenticated by sequencing) using LipofectamineLTX and PLUS reagent (Invitrogen). Efficiency, confirmed by EGFP expression, was greater than 85% at 48–72 hours after transfection. Images were acquired using a Nikon PlanFluor 10× (0.5 NA), PlanApo 20× (0.75 NA) or PlanApo 63× (1.4 NA) objective.

Migration and scratch wound assays

MDA-MB-231 migration assays were performed as previously described (Beauvais et al., 2004) and data plotted as the mean percentage of cells that migrated relative to the paired growth factor-stimulated (EGF or IGF alone) cohort. HUVECs were grown to confluence in six-well plates pre-coated with VN. Cells were serum-starved for 24 hours and a 'scratch wound' was then created by running a sterile pipette tip across the monolayer to remove a swatch of cells. Images were recorded immediately after wounding (T_0). Cells were then stimulated with 20 ng/ml VEGF-A165 and images recorded again 18 hours later using a Nikon PlanFluor 4× (0.13 NA) objective. Cell migration was quantified using image analysis of 12 randomly selected fields of denuded area. The mean wound area is expressed as percentage of wound closure (% closure) from three identically treated wells and two independent experiments using the equation: $\%\ \text{closure}=[1-(T_{18}/T_0)]\times100$, where T_0 is the wounded area at 0 hours and T_{18} is the wounded area after 18 hours.

Flow cytometry

WOW1 staining (in the presence or absence of inhibitors) and cell scanning were conducted as previously described (Beauvais et al., 2009). To cluster mouse Sdc1, human Sdc1 or IGF1R, cells were incubated (15 minutes each at 37°C) with 1 μg/ml primary mAb 281.2, B-A38 or 3B7 (or rat IgG2A or mIgG1 as isotype controls), respectively, followed by a goat secondary antibody. Alternatively, cells were stimulated with 100 ng/ml of IGF. To detect activated IGF1R, cells were permeabilized and stained with Alexa-Fluor-647-conjugated IGF1R phospho-tyrosine 1131 (pY1131)-specific antibody, mouse mAb K74-218 in CMF-PBS containing 1% heat-denatured BSA and 0.1% w/v saponin (Sigma). Mean fluorescent intensity (MFI) of triplicate samples for each cohort was used to ascertain the levels of activated integrin and IGF1R.

Immunoprecipitation

Immunoprecipitation of Sdc1 or IGF1R [using rabbit polyclonal antibody CBL257 or mouse mAb JBW902 (Millipore)], with or without competing SSTN, was performed as previously described (Beauvais et al., 2009). For precipitations using purified components, 'bait' proteins immobilized to beads (GST–mouse-S1ED protein to glutathione agarose or IGF1R immobilized to mAb-3B7-coated GammaBind G-Sepharose) were incubated with purified integrin alone, integrin plus IGF1R or GST–S1ED, respectively, in the presence of competitive $SSTN_{92–119}$ or non-competitive $SSTN_{94–119}$ peptide in sterile PBS containing the protease inhibitor cocktail for 4 hours at 4°C. In biotin-label transfer assays, biotinylated SSTN [active 92–119 or inactive 94–119 peptide labeled as previously described (Beauvais et al., 2009)] was incubated with HMEC-1 cells in culture and crosslinked to interacting cell surface proteins under UV. After lysing the cells and reversing the crosslinks with DTT, biotin-labeled proteins were captured using anti-biotin agarose (Sigma) and eluted in 0.1 M glycine and 0.15 M NaCl, pH 2.4.

ImmobilonP blots were probed with either alkaline-phosphatase-conjugated streptavidin, rabbit anti-β3-integrin 'Fire and Ice' (10 μg/ml), polyclonal anti-β5-integrin, anti-αv-integrin (1:1000) or anti-IGF1Rα (1 μg/ml), mouse anti-β1-integrin mAb N29 (2 μg/ml), anti-αv-integrin mAb 3F12 (10 μg/ml), anti-talin mAb TA205 (2 μg/ml), anti-HA mAb 12CA5 (2 μg/ml) or anti-ΔHS mAb 3G10 (1 μg/ml), anti-Sdc1 mAbs B-A38 or 281.2 (1 μg/ml), mouse anti-IGF1Rβ mAb 7G11 or 1-2 (1 μg/ml), IGF1Rα mAb 1H7 (Serotec) or anti-phospho-IGF1R mAb JY202 (2 μg/ml) followed by an alkaline-phosphatase-conjugated secondary antibody. Wherever possible, blots were stripped and re-probed for the presence of other receptors (e.g. IGF1Rβ and Sdc1, integrin and Sdc1 or integrin and IGF1Rα). When this was not possible (because of nearly equivalent relative molecular masses, e.g. IGF1Rβ and integrin), duplicate experimental samples were run on separate blots and then probed individually. Visualization of immunoreactive bands was performed using ECF reagent (Amersham Pharmacia, Piscataway, NJ) and scanned on a Storm PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

All statistical analyses were performed as previously described (Beauvais et al., 2009).

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