Displacement of the β cytoplasmic domain recovers focal adhesion formation, cytoskeletal organization and motility in swapped integrin chimeras

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Summary

Integrin-mediated 'outside-in' signaling requires the transmission of a conformational change from the extracellular domains to the cytoplasmic domains. Although one component of this conformational change is the separation of the α and β cytoplasmic domains, it is not clear how this separation could result in the initiation of downstream signals necessary for focal adhesion (FA) formation. To address this question, we used a swapped integrin heterodimer, in which the extracellular domains of the α and β chains were attached to their opposing transmembrane and cytoplasmic domains. This receptor was able to bind ligand normally, but could not promote FA formation. We then displaced the β cytoplasmic domain with either a duplication of its membrane-proximal region or an unrelated α-helical spacer. This displacement partially restored FA formation in these swapped receptors and rescued other aspects of integrin-mediated signaling, including cytoskeletal organization, motility and several tyrosine-phosphorylation-dependent signals. We suggest that separation of the cytoplasmic domains leads to alteration of the secondary structure of the distal β tail, which initiates downstream signals leading to cytoskeletal reorganization.

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Key words: Integrin, Focal adhesions, Cell motility, Outside-in signals

Introduction

Integrins are a class of transmembrane heterodimeric glycoproteins that serve as extracellular matrix (ECM) receptors. The integrin family is ubiquitously expressed, although the distinct α and β subunits that comprise the receptor can be cell-type specific and, in turn, confer differences in the binding capability of a cell for particular ECM substrates. In addition to their ECM-binding role, integrins also cluster an array of cytoplasmic proteins at plaques called focal adhesions (FAs), which provide anchor points for the actin cytoskeleton and serve as a signaling hub for kinases, adaptor proteins and GTPases (Hynes, 2002).

Recent integrin structural analysis indicated that dissociation of the cytoplasmic domains of the α and β subunits altered the affinity of the receptor for ligand (Takagi et al., 2001). In complementary studies, it was noted that ligand-bound integrins displayed physically separated cytoplasmic tails (Xiao et al., 2004). These results confirmed several in vitro findings suggesting that a cytoplasmic conformational change in response to ligand was important for the propagation of integrin-mediated adhesion signals. These included the observations that specific ligand interaction, and not simple antibody clustering, was essential for outside-in signaling to recruit FA proteins, and that particular cytoplasmic FA proteins could mediate inside-out signals to alter integrin extracellular structure (Miyamoto et al., 1995; Calderwood et al., 1999).

The membrane-proximal portions of the α and β cytoplasmic domains interact through a salt bridge and might also form a coiled-coil-like structure (Hughes et al., 1995; Williams et al., 1994). Disruption of this connection by separation of the integrin cytoplasmic domains in response to ECM binding might simply expose these regions, enabling association with other proteins necessary for FA formation. Alternatively, or in addition, disruption of the cytoplasmic α-β interaction might allow the membrane-proximal region to adopt a different secondary and/or tertiary conformation, which is then propagated to the distal tails. Of particular interest in the transmission of this signal is the β cytoplasmic domain, since proteins that link integrins to the actin cytoskeleton have been found to bind to this region, and numerous studies have implicated it as the main engine of FA formation (Briesewitz et al., 1993b; Calderwood and Ginsberg, 2003; David et al., 1999; LaFlamme et al., 1992).

Previous work from our laboratory characterized a mutant integrin heterodimer in which the extracellular regions of the human α_1 and the chicken β_1 chain were swapped onto their opposing transmembrane and cytoplasmic domains (Fig. 1A,B) (Briesewitz et al., 1995). When expressed in NIH3T3 cells (SW1 cells), these subunits selectively associated with one another, were expressed on the cell surface and bound their ligand, collagen IV, approximately equivalently to the wild-type receptor. However, despite normal ligand-binding

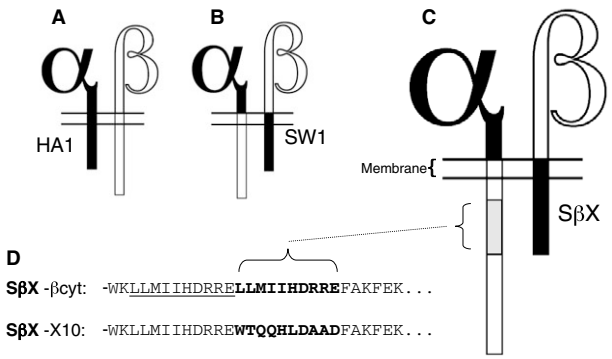

Fig. 1. Representations of the human α_1 and chicken β_1 collagen IV receptor and the swapped integrin chimera. (A) HA1, or wild-type receptors, express full-length α_1 integrin. (B) SW1 receptors have the extracellular regions swapped onto their opposing transmembrane and cytoplasmic domains (Briesewitz et al., 1995). (C) SβX receptors are altered from the SW1 chimera by extension of the β cytoplasmic domain by 10 residues. (D) Two constructs with 10-residue insertions (bold) were engineered: SβX-βcyt, a duplication of the first 10 residues of the β cytoplasmic domain (underlined); and SβX-X10, a random sequence predicted to be α-helical.

properties, the swapped integrin receptor was defective in mediating the bulk of adhesion signals including FA formation (Briesewitz et al., 1995). The most likely explanation for the phenotype of this chimeric receptor was that cytoplasmic domains were not activated by the ligand-dependent conformation change that would have occurred in the wild-type receptor upon ECM binding. Consequently, we were interested in assessing the biological response (particularly the phenotypic, biochemical and motile behavior of cells) to adhesion mediated by the swapped receptor with an altered cytoplasmic domain structure that mimicked a ligand-induced conformational change. Specifically, we wanted to expose part of the β cytoplasmic domain that might otherwise be buried or shielded by the α cytoplasmic domain by inserting a 10-residue extension immediately downstream of the transmembrane region.

Using this altered receptor, we wanted to determine whether aspects of adhesion-mediated signaling and cytoskeletal organization could be rescued in comparison with the SW1 integrin. In particular, we wanted to analyze FA generation and signaling with the SW1 receptor, and whether any defect could be recovered by the separation of the α and β cytoplasmic domains of the integrin. Furthermore, given the fundamental role of FA formation in migration, we wanted to assess whether motility was affected by the dissociation of the α and β cytoplasmic tails. These studies have implications for understanding the interactions between integrin-associated proteins and the receptor, and how ligand binding regulates these interactions during cell adhesion and migration.

Results

In order to mimic the physical separation of the α and β cytoplasmic domains in response to ligand, an extension was inserted in the β cytoplasmic domain of a swapped integrin chimera (designated SβX) that preserved receptor assembly (Fig. 1C). Two mutations were engineered (Fig. 1D). In one, the first ten residues of the β cytoplasmic domain were duplicated immediately downstream of their normal location (βcyt). This included the region that is probably masked during salt bridge formation with the opposing region of α_1. We reasoned that this insertion in the β_1 cytoplasmic domain might mimic the exposure of this region during outside-in signaling. To test the effect of changing the secondary/tertiary structure of the distal β_1 cytoplasmic domain, we also inserted an unrelated ten-residue spacer in the same location (X10). Both of these spacer sequences were predicted to adopt an α-helical conformation in the context of the entire β_1 cytoplasmic domain by a neural-network-based, secondary-structure-prediction algorithm (Rost and Sander, 1993; Rost et al., 1994). These constructs were stably transfected into NIH3T3 cells and surface expression of the chimeric heterodimer confirmed by flow cytometry with an antibody specific for the extracellular domain of human α_1 integrin (Briesewitz et al., 1995) (Fig. S1, supplementary material).

Biochemical signaling mediated by swapped integrin chimeras

Although SW1 receptors bound collagen IV approximately normally, they were unable to assemble FAs and actin stress fibers (Briesewitz et al., 1995). FAs are plaques incorporating a variety of proteins that mediate the connection between the integrin and the actin cytoskeleton. Among the molecules present in FAs are kinases and adaptor proteins, some of which become phosphorylated at tyrosine residues upon adhesion (Hynes, 2002). However, the SW1 integrin had also been shown to be defective in its ability to induce adhesion-dependent phosphorylation signals (Briesewitz et al., 1995). At least three prominent FA proteins become tyrosine phosphorylated upon adhesion: the tyrosine kinase focal adhesion kinase (FAK), and the adaptor proteins Cas and paxillin (Chodniewicz and Klemke, 2004; Turner, 2000; Mitra et al., 2005). We initially wanted to determine whether each of these proteins was defective in phosphorylation in SW1 cells. Subsequently, if any of these proteins were phosphorylated normally in the 'activated' (ligand-bound) receptor-mimic SβX, then we may be able to reveal specific signaling pathways activated by integrins.

FAK performs important regulatory and catalytic roles in adhesion and is activated by another non-receptor tyrosine kinase, Src (Klinghoffer et al., 1999; Sieg et al., 1999). Src binds through its SH2 domain to the autophosphorylated tyrosine residue Y397 of FAK and then phosphorylates and activates the catalytic site of FAK, Y576/577 (Cary et al., 2002). Interestingly, on collagen IV, SW1 supported phosphorylation of FAK at Y397 and at the kinase domain site, Y576 (Fig. 2A). The level of FAK phosphorylation mediated by the SW1 receptor was similar to the SβX and the wild-type integrin (Fig. 2A). FAK activation at both Y397 and Y576 is crucial for the propagation of downstream signals upon adhesion and for normal cell morphology and migration (Cary et al., 2002; Sieg et al., 1999). However, in SW1 cells, despite normal FAK activity, the phosphorylation of two FAK substrates, Cas and paxillin, was defective (Fig. 2B). By contrast, the SβX receptor supported the phosphorylation of the adaptor protein paxillin on collagen IV (Fig. 2B). However, Cas phosphotyrosine levels remained reduced with the SβX receptors, demonstrating that the extension of the β cytoplasmic domain was not sufficient to rescue all aspects of

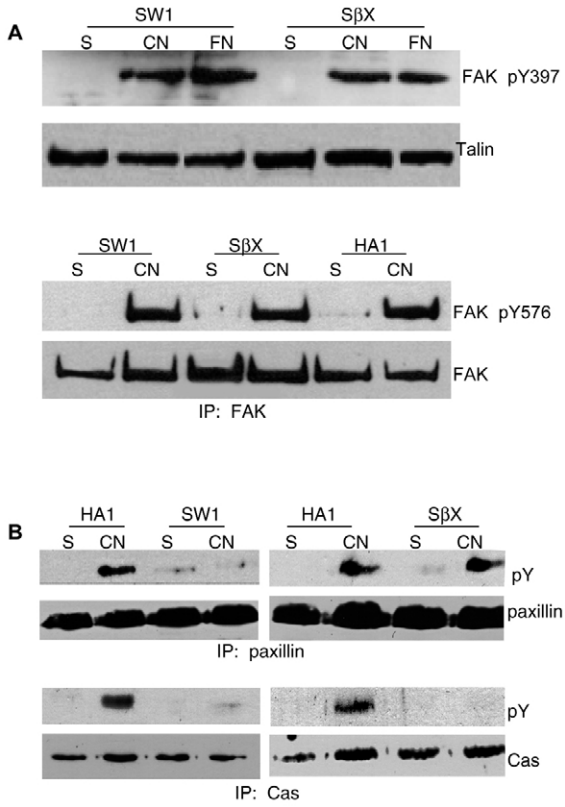

Fig. 2. Analysis of FA protein phosphotyrosine levels. Biochemical analysis of FA protein phosphotyrosine levels in cells expressing wild-type human α1 (HA1) or the swapped integrin chimers (SW1 or SβX). Cells were kept in suspension for 1 hour and allowed to spread on collagen IV (CN) or fibronectin (FN) for 1 hour, and phosphotyrosine levels were compared with the suspension control (S). (A) Phosphorylation of FAK at Y397 and Y576 were detected with site-specific pY antibodies (Biosource). Lysates were used neat (50 μg) for pY397 blots or immunoprecipitated (IP; 500 μg) with anti-FAK mAb (Upstate) for pY576 blots. Membranes were reprobed with anti-FAK (Transduction) or anti-talin (Sigma) mAbs. (B) Total phosphotyrosine levels in immunoprecipitates of paxillin or Cas. Both SW1 and SβX cells mediate normal FAK phosphorylation but are defective in Cas phosphorylation. SβX cells mediate wild-type levels of paxillin phosphorylation.

signaling, as well as reinforcing previous findings that Cas and paxillin function through divergent signaling pathways (Chodniewicz and Klemke, 2004; Turner, 2000).

Morphology of cells adhering with integrin chimeras

Having established that the SβX integrin mediated phosphorylation of FA proteins downstream of FAK, we wished to determine if the receptor could rescue FA formation and cytoskeletal organization. Surprisingly, in comparison with SW1 cells, a dramatic change in morphology was observed in SβX cells on collagen IV (Fig. 3A,B). The cells formed large lamellipodia similar to structures observed at the leading edge of a migrating cell (Pollard and Borisy, 2003). Many of the cells generated multiple membrane ruffles extending in different directions from the cell body. These veils of membrane displayed extensive cortical actin organization anchored by small SβX integrin-containing FA-like structures that formed radially to the lamellipod (Fig. 3B).

Importantly, the formation of FA-like structures with the SβX cells was consistent with the observed biochemical results. Given the wild-type status of FAK Y397 phosphorylation in both SW1 and SβX cells, it was not unexpected that Src had subsequently phosphorylated FAK Y576, thus activating the kinase (Cary et al., 2002). However, the fact that FAK was active in both cell lines and yet paxillin was only phosphorylated in SβX cells, strongly suggested that recruitment of paxillin was defective with SW1 and rescued with SβX receptors. This result was confirmed by morphological observations that paxillin was also evident in these radial, SβX integrin-containing, FA-like plaques (Fig. 4A). Conversely, the inability of the SβX chimera to mediate Cas phosphorylation indicated that the exposed β cytoplasmic domain was still partly defective in the recruitment of FA proteins compared with the wild-type $\alpha_1\beta_1$ integrin. Once again, the biochemical data was confirmed by morphological analysis indicating that Cas was capable of localizing to FAs formed by wild-type integrins, but was unable to localize to FA-like structures in SβX cells adhering to collagen IV (Fig. 4B). In addition to paxillin, two other FA proteins, vinculin (Fig. 5B) and FAK (Fig. 4A,B), were also evident in these FA-like plaques. This finding was consistent with the ability of the SβX receptor to recruit and phosphorylate paxillin, as paxillin has been shown to interact through its LD domains with both proteins at their respective FA targeting sequence (Tachibana et al., 1995; Wood et al., 1994). Furthermore, the persistent defect in Cas phosphorylation corroborated morphological data from $Cas^{-/-}$ fibroblasts, which also form lamellipodial-like structures, albeit not as strikingly as SβX cells (Honda et al., 1998).

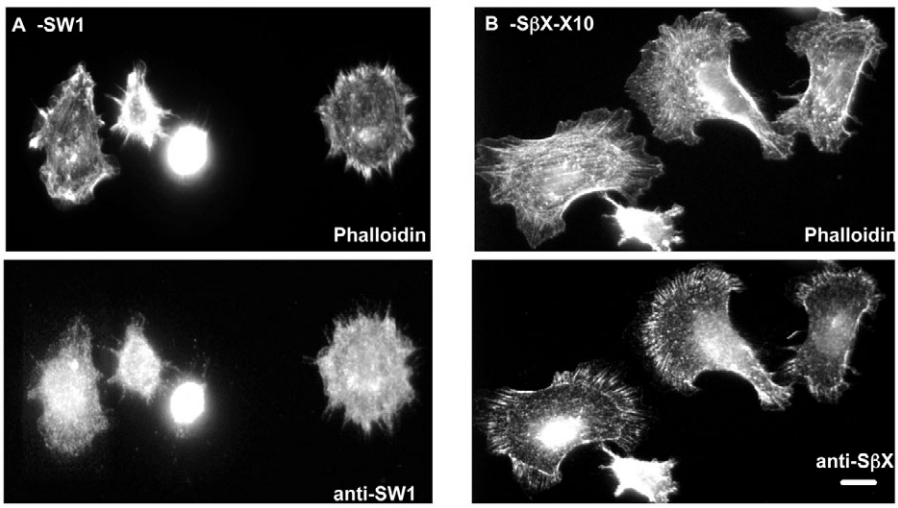

Fig. 3. Morphology of SW1 and SβX cells on collagen IV. Cells were allowed to spread on collagen IV for 1 hour and SW1 (A) and SβX-X10 (B) cells stained for F-actin with Rhodamine-Phalloidin or with antibody to the α_1 integrin extracellular domain to identify the chimeric receptors. Bar, 10 μm.

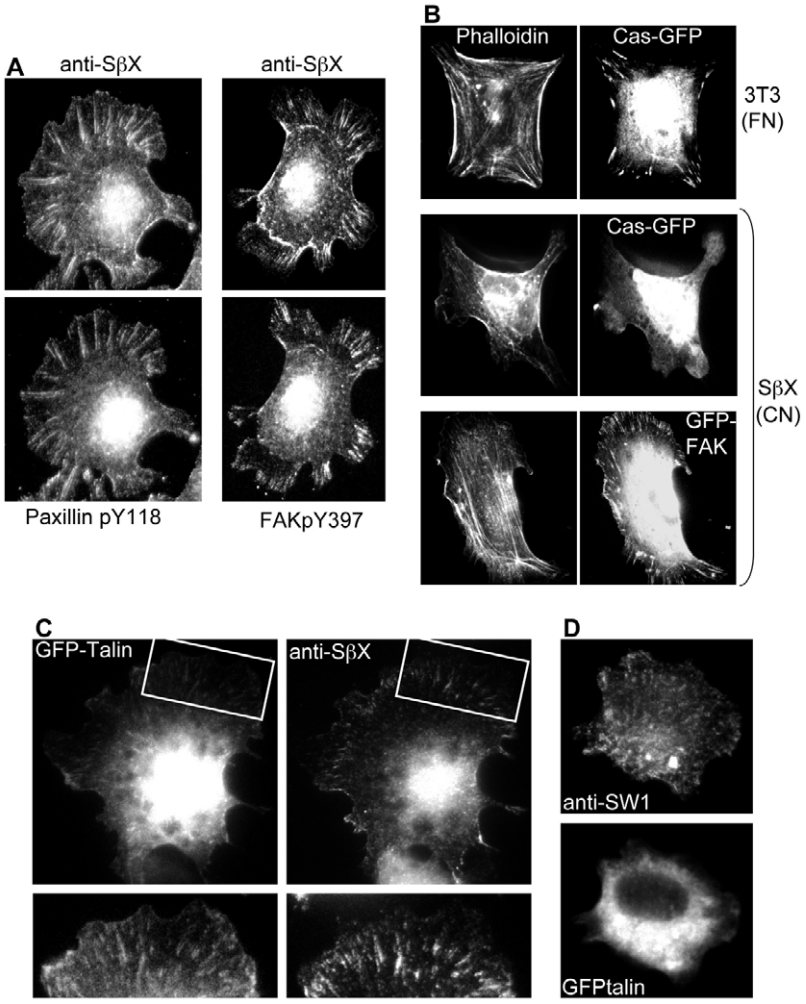

Fig. 4. Localization of FA proteins to the SβX receptor in cells spreading on collagen IV. (A) Cells expressing SβX-βcyt were allowed to spread on collagen IV and stained for the SβX receptor (anti-α_1 integrin) and with antibodies to either paxillin pY118 or FAK pY397. (B) Cells expressing SβX-βcyt transiently transfected with either Cas-GFP or GFP-FAK were allowed to spread on fibronectin (FN) or collagen IV (CN) and stained for F-actin with Rhodamine-Phalloidin. Spreading on fibronectin indicates that Cas-GFP was able to localize to FAs generated with wild-type integrins whereas cells transfected with GFP-FAK verify that transiently transfected fluorescently tagged FA protein constructs were able to localize to radial FA-like structures formed by SβX cells on collagen IV. SβX-βcyt (C) or SW1 (D) cells were transiently transfected with GFP-talin, allowed to spread on collagen IV and stained for the chimeric receptors. Lower panels in C are a higher magnification of the regions outlined in images of the whole cell. Talin, paxillin and FAK, but not Cas, localize to FA-like structures formed with SβX cells on collagen IV.

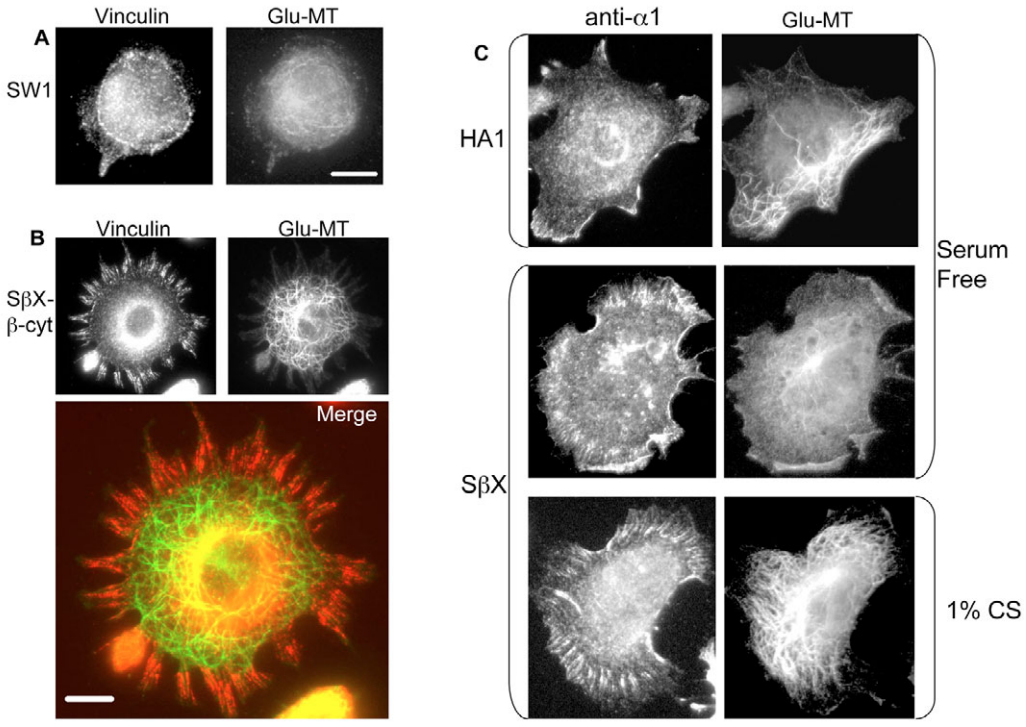

Fig. 5. Formation of stable microtubules in cells expressing HA1, SW1 or SβX integrin. (A,B) SW1 and SβX (SβX-β-cyt) cells were allowed to spread on collagen IV and stained for vinculin to identify FA structures and Glu-tubulin to reveal stabilized microtubules (Glu-MT). Bar 10 μm. (C) HA1 cells and SβX cells were allowed to spread on collagen IV with or without 1% calf serum (CS) and stained with anti-α_1 (anti-α1) integrin to identify the HA1 and SβX integrins, and Glu-MT to reveal stabilized microtubules. Only in the presence of serum do SβX, but not SW1, cells form stable microtubules on collagen IV.

In addition to FAK and paxillin, we were particularly interested in determining whether another FA protein, talin, might also be localized to the FA-like structures present in SβX cells on collagen IV. Talin is a ~270 kDa protein that is thought to form the crucial initial connection to the β cytoplasmic tails of integrins upon ligand binding. Additionally, talin also binds to actin, providing the link between integrins and the cytoskeleton (Calderwood and Ginsberg, 2003; Jiang et al., 2003; Tadokoro et al., 2003). The presence of enhanced actin organization in SβX cells suggested that talin might also be localized to the FA-like structures formed on collagen IV. As expected, when SβX cells were transfected with GFP-talin, the fluorescently labeled protein was observed in SβX integrin-containing FA-like structures (Fig. 4C). By contrast, the expression of GFP-talin in SW1 cells did not promote the formation of adhesions on collagen IV (Fig. 4D).

Notably, for all adhesion events tested on collagen IV, both the X10 and the βcyt insertion in the SβX receptor (Fig. 1) mediated an identical phenotype, with both signaling properties and cell morphology rescued in comparison with the SW1 receptor (Fig. 3B and Fig. 4A). Hence, the effects seen with the SβX receptors were probably induced by exposure of sequences downstream of the insertion, or changes to the secondary structure of the cytoplasmic domain of the receptors, and not simply due to the duplicated β sequence or the exposure of proximal sequences facilitating protein-protein interactions.

Having identified a substantial enhancement of actin organization with the rescued receptor, we wanted to determine whether other cytoskeletal structures had an analogous improvement in organization. In fibroblasts, there are at least two types of microtubules: dynamic microtubules that have half lifes of fewer than 10 minutes; and stable microtubules that can endure for hours (Palazzo et al., 2001). Of the attributes associated with microtubule stabilization, one of the best characterized is the post-translational modification of α-tubulin by detyrosination, exposing a glutamate residue (Bulinski and Gundersen, 1991). This marker of microtubule stabilization (Glu-MT), which is detectable by specific antibodies, is dependent on adhesion, through FAK, and on the Rho GTPase, through its effector protein, mDia1 (Palazzo et al., 2001; Palazzo et al., 2004).

Previous data suggested that Glu-MT formation required tyrosine phosphorylation of FAK, but not Cas or paxillin, to bring activated Rho in proximity to their effectors (Palazzo et al., 2004). We therefore expected that the SW1 receptor, which supported FAK phosphorylation, might induce Glu-MT formation despite their inability to organize the actin cytoskeleton. Surprisingly, SW1 cells remained unable to promote Glu-MT formation, whereas the SβX receptor was able to support stabilized microtubules on collagen IV (Fig. 5A,B). Therefore, FAK phosphorylation, although necessary for Glu-MT formation (Palazzo et al., 2004), was not sufficient for this process, and the SβX chimera, but not SW1, delivered the additional signal (or signals) required for microtubule stabilization, probably through formation of FA-like structures.

The improvement in organization of the cytoskeleton and the formation of lamellipodia in SβX cells indicated that, apart from the recruitment and phosphorylation of paxillin, mimicking the receptor-ligand interaction might also cause activation of two members of the Rho family of small GTPases, Rac and Rho. GTP-bound Rac has been shown to promote lamellipodia formation and, as mentioned, Rho activation is necessary for stabilization of microtubules (Cook et al., 1998; Nobes and Hall, 1995). Consequently, we wanted to assess the level of adhesion-dependent activation of Rac and Rho for SW1 and SβX receptors on collagen IV (del Pozo et al., 2000; Ren et al., 1999). Initially, we confirmed that the wild-type $\alpha_1\beta_1$ integrin mediated Rac activation on collagen IV similar to the endogenous $\alpha_5\beta_1$ integrin on fibronectin (Fig. 6A,C) using glutathione *S*-transferase (GST) pull-down assays (Glaven et al., 1999; Ren et al., 1999). Subsequently, Rac and

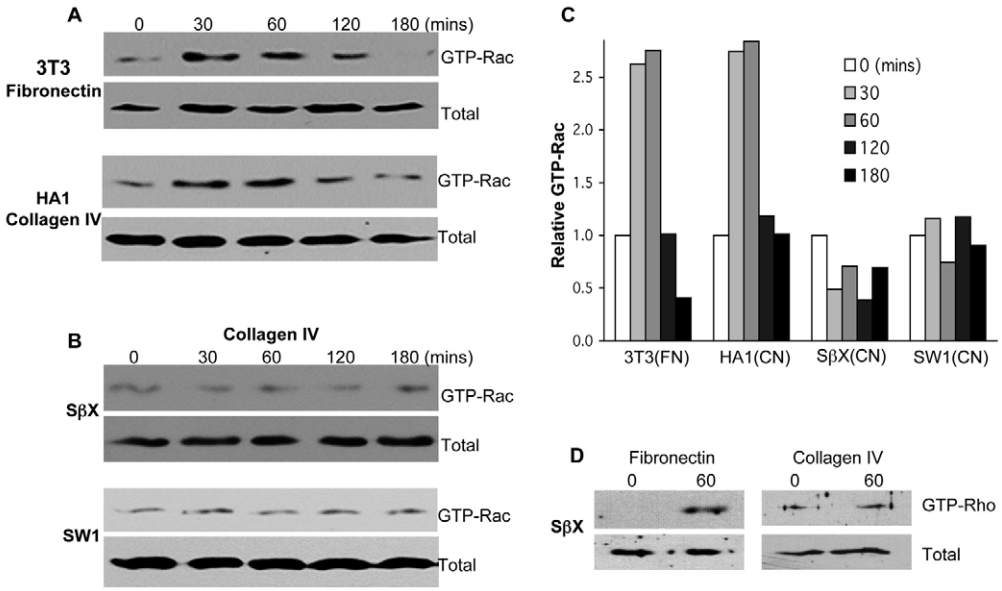

Fig. 6. Rac and Rho activation by integrins. Activation of the small GTPases Rac and Rho in response to adhesion in parental NIH3T3 cells on fibronectin and cells expressing HA1, SW1 or SβX on collagen IV (CN). Cells were maintained in 1% serum overnight, placed in suspension for 1 hour and plated in 1% serum (Ren et al., 1999) on fibronectin or collagen for the times indicated. (A,B) Lysates were incubated with GST-PBD beads for 30 minutes and bound GTP-Rac detected by western blotting. Total cell lysates probed for Rac demonstrated equal amounts of Rac. (C) Graphical representation of the relative increase in GTP-Rac for each of the experiments. Relative GTP-Rac was normalized around levels obtained at 0 minutes adhesion time and adjusted for total Rac loading. (D) SβX cells were plated on fibronectin or collagen IV, lysates were incubated with GST-Rhotekin-RBD and bound GTP-Rho detected by western blotting. Total cell lysates probed for Rho demonstrate equal amounts of protein. Activation of Rac and Rho in response to collagen IV is normal for wild-type α_1-expressing cells but defective for swapped integrin chimeras.

Rho activation was analyzed in SW1 and SβX cells on collagen IV and, unexpectedly, neither Rac nor Rho was activated in an adhesion-dependent manner with either swapped integrin chimera (Fig. 6B-D).

Our findings that SβX cells activated FAK and supported microtubule stabilization but did not activate Rac or Rho was in contrast to the requirement for adhesion-derived stimulation of both the kinase and the GTPase in Glu-MT formation (Cook et al., 1998; Palazzo et al., 2004). However, the morphological experiments reported here were performed in the presence of serum, which can also activate Rho-family GTPases (Cook et al., 1998; Ren et al., 1999). We wondered whether, in SβX cells, serum-stimulated Rho activation was compensating for the lack of adhesion-mediated GTP-Rho to provide the signal required for Glu-MT. Initially, we confirmed that HA1 cells, which supported adhesion-dependent GTPase activation (Fig. 6), were able to sustain stabilized microtubules on collagen IV (Fig. 5C). Subsequently, when we assessed Glu-MT formation with the SβX receptor in the absence of serum, we found that, although the cells spread and formed FA-like structures, they were unable to support stabilized microtubules. Consequently, SβX cells still required a serum-derived signal (Fig. 5C), presumably the activation of Rho by lysophosphatidic acid (LPA) (Cook et al., 1998), for Glu-MT formation.

FAs are thought to provide a focal point for signaling between kinases, GTPases and adaptor proteins. Paxillin, an FA protein both phosphorylated and localized to FA-like structures in SβX, has been shown to bind through a complex of proteins to the Rac and Cdc42 effector PAK (Turner, 2000). Furthermore, paxillin can bind directly to tubulin (Herreros et al., 2000) and indirectly to F-actin (Nikolopoulos and Turner, 2000). In the case of SβX cells, once small focal complexes containing vinculin as well as phosphorylated FAK and paxillin were initiated by the SβX integrin chimera, this accumulation of proteins might have served as a signaling intersection for serum-activated Rho and Rac to effect enhanced cytoskeletal organization. This supports the assertion that paxillin might function as a fulcrum for crosstalk between GTPases, cytoskeletal proteins and integrins (Brown and Turner, 2004; Turner, 2000). However, the fact that actin organization was not completely rescued with SβX receptors suggests that reciprocal adhesion-mediated activation of Rho GTPases (which did not occur in SβX cells) was necessary to fully restore wild-type cytoskeletal organization and FA formation.

Cell spreading and migration with wild-type α_1 integrin and swapped chimeras

The dramatic change in morphology of the SβX cells on collagen IV compared with SW1 cells indicated that cell spreading was enhanced by separation of the α and β cytoplasmic domains of the receptors. To evaluate this change, we measured the two-dimensional cell area during spreading on collagen IV by phase-contrast microscopy. Interestingly, the increase in area (±s.e.m.) of SβX cells (320±35 μm^2, n=10) after 2 hours of spreading, although less than with the HA1 receptor (440±43 μm^2, n=16) was more than twice that recorded for SW1 cells (150±15 μm^2, n=13).

In spreading cells, in order for the cell to adhere and flatten, integrins must bind to the ECM and form new FAs. In an adherent cell, the formation of new adhesions at the leading edge is also crucial to providing forward motility. The fact that spreading was enhanced in the SβX cells and that, unlike SW1 cells, they formed FA-like structures, suggested that migration might be improved with the activated-receptor mimic. Furthermore, given the improved signaling capability and cytoskeletal organization with the SβX receptor, and in particular the obvious phenotypic similarity to a cell migrating with a lamellipodia at the leading edge (Pollard and Borisy, 2003), we analyzed motility in these cells compared with the SW1 and wild-type HA1 cells.

To accomplish this, we observed dynamically the random movement of cells sparsely plated on collagen IV over 10-12 hours. We wanted to study random motility, rather than directed migration into a wound, to minimize or exclude from analysis two potential problems encountered in wound healing migration: cell-cell contact and adhesion to areas where other cells might have previously deposited fibronectin. Using this technique, we initially examined random migration of parental NIH3T3 cells on fibronectin using their endogenous receptor. Interestingly, the speed of random migration of NIH3T3 cells on fibronectin was dramatically lower (Fig. 7) than the velocity of fibroblasts in wound healing (Liao et al., 1995; Etienne-Manneville and Hall, 2001) (M.A.P. and E.E.M., unpublished). This result clearly indicated that these two methods for analyzing cell migration were measuring distinctly different processes.

Unexpectedly, in random migration, the velocity of HA1 cells on collagen IV was more than double their speed on fibronectin (Fig. 7), although still substantially less than migration in wound healing. However, random motility of SW1 cells was significantly lower than HA1 cells on collagen IV (Fig. 7). This motility defect was consistent with their inability to form FAs, organize the cytoskeleton or support adhesion-dependent signaling pathways. By contrast, for the SβX receptor, random migration on collagen IV was similar to the wild-type α_1 integrin (Fig. 7). Hence, displacement of the β cytoplasmic domain in the swapped chimeric receptor had rescued motility as well as enhanced cytoskeletal organization and adhesion-related signaling. In addition, the persistence of

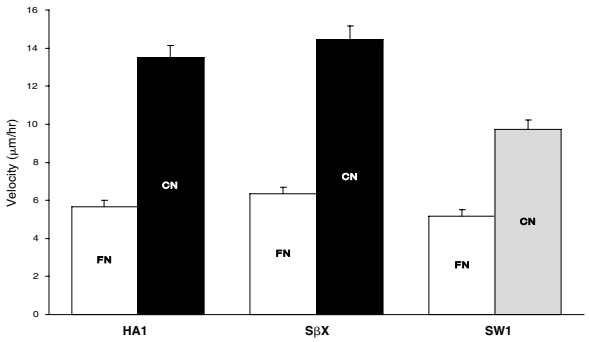

Fig. 7. Random migration of cells expressing HA1, SW1 or SβX on collagen IV or fibronectin. Cells were sparsely plated onto glass-bottomed culture dishes coated with fibronectin (FN) or collagen IV (CN) and allowed to spread for 1 hour. Dishes were then placed on a heated microscope stage and images captured every 20 minutes for 10-12 hours. Data are average velocity measured by tracking cell nuclei of at least 10 cells from each of two (HA1 and SW1) or three (SβX) experiments (±s.e.m.). Bars with different shadings are significantly different from each other (Student's t test, P<0.01).

movement (average distance between start and finish points) was not significantly different between the wild-type (6.6±0.6 μm/hr, *n*=26) and chimeric integrin (6.0±0.5 μm/hr, *n*=38), indicating that the motility mediated by the SβX receptor was not more chaotic. Importantly, the increase in motility with the SβX receptor was not due to increased expression because surface levels of chimeric and wild-type integrins were similar (Fig. S1, supplementary material) (Briesewitz et al., 1995).

The distinct difference in the random migration of HA1 cells on collagen IV compared with fibronectin was unexpected because, in all assays of integrin function previously examined, the behavior of HA1 cells on the two substrates had been similar (Figs 2 and 6) (Briesewitz et al., 1993a; Briesewitz et al., 1995) (M.A.P. and E.E.M., unpublished). Furthermore, surface expression of exogenous α_1 and endogenous α_5 integrin was similar (Fig. S1, supplementary material), indicating that the discrepancy observed in random migration between the two substrata was unlikely to be due to over-expression of the α_1 integrin in the stable cell line. A more likely explanation is that a property of the $\alpha_1\beta_1$-collagen IV interaction, such as receptor-ligand affinity, a different epitope density, or differences in coating efficiency between fibronectin and collagen IV, caused the increase in random migration velocity compared with the motility of the cells on fibronectin. This result was important for two reasons. First, the increased velocity of HA1 cells on collagen IV improved the degree of discrimination between wild-type α_1-expressing lines and the chimeras and, consequently, this ligand-receptor might be useful for analysis of motility in fibroblasts. Second, this result indicated that different receptor-ligand combinations can support equivalent levels of biochemical activity but mediate dramatically different motile behavior, a finding that might have significance in vivo where receptors and substrates may be more readily interchangeable.

Discussion

Previous work in our laboratory on integrin dimerization and signaling lead to the development of a receptor in which the extracellular domains of the α_1 and β_1 subunits were swapped. This chimeric collagen receptor dimerized, was expressed at the cell surface and bound ligand effectively, approximately comparable with the wild-type α_1 integrin. Furthermore, in response to ligand, the receptor supported FAK phosphorylation. However, much like antibody clustering of integrins (David et al., 1999; Miyamoto et al., 1995), no other signal transduction events occurred and higher order actin organization was not supported (Briesewitz et al., 1995).

We attempted to mimic a conformational change in the cytoplasmic domain of the swapped receptor by engineering an extension in the β cytoplasmic chain. Interestingly, the behavior of this receptor, SβX, was partially consistent with integrin-ligand occupancy (Miyamoto et al., 1995). Paxillin phosphorylation upon adhesion to collagen IV was normal, the actin cytoskeleton displayed enhanced organization and stable microtubules were formed. Furthermore, the spread cell area was dramatically increased, nascent focal contacts were formed and FAK, vinculin and paxillin were recruited to these structures. In addition, migration of SβX cells was also normal. However, signaling to the small GTPases Rac and Rho was defective.

Despite the integrin chimeras not activating small GTPases, FA-like structures formed and cytoskeletal organization was enhanced, both of which are morphologies that require adhesion signals as well as activated Rho and Rac (Nobes and Hall, 1995; Palazzo et al., 2004). Importantly, experiments reported here were performed in the presence of serum, which also activates small GTPases. This fact, combined with the ability of paxillin to interact with effectors of Rac and Cdc42, as well as cytoskeletal components (Turner, 2000), presents the possibility that paxillin can mediate cross-talk between GTPases, cytoskeletal proteins and the adhesion apparatus (Brown and Turner, 2004).

Integrins are allosteric receptors and, consequently, comprehensively mimicking their complex structural alterations in response to ligand in the context of the swapped integrin chimera is inherently difficult. The SβX integrin was able to bind ligand effectively and mimic 'outside-in' signaling to a significant degree. However, wild-type integrins have a bi-directional signaling capability (Hynes, 2002), which might not occur appropriately in the swapped receptors. Consequently, it was possible that, despite the recruitment of a subset of FA proteins to the nascent adhesion complexes, the receptor was unable to transmit an 'inside-out' signal and undergo transformation to a higher order affinity state for ligand. Indeed, the SW1 receptor was approximately two thirds as effective as the wild-type integrin in binding ligand (Briesewitz et al., 1995), suggesting the possibility that, after initial integrin-ligand interaction, there was some modest improvement in adhesiveness due to inside-out signaling in the wild-type integrin but not in the chimeric receptors. However, it was equally possible that a further conformational change in the cytoplasmic domains (such as lateral separation) or physical realignments of the transmembrane domains was also required to truly mimic integrin-ligand binding and fully rescue signaling (Takagi et al., 2001; Luo et al., 2005).

In this work, we demonstrate that exposure of the distal β cytoplasmic domain in a swapped integrin chimera is sufficient to recapitulate many aspects of ligand-dependent signaling, including activation of several downstream signaling components, reorganization of actin and microtubules, FA formation, spreading and migration. Prior work on integrin-mediated signaling demonstrated that the proximal α and β cytoplasmic domains are associated when integrin are in the resting state, and that activation leads to their separation (Xiao et al., 2004). From these data, it was not clear if cytoplasmic domain separation results in exposure of proximal sequences necessary for protein-protein interactions and/or the adoption of a novel secondary/tertiary conformation by the distal β tail. Our work suggests that presentation of the distal β tail away from the proximal α cytoplasmic domain, presumably allowing it to adopt a novel structural conformation, suffices to recapitulate many aspects of integrin-mediated signaling and elicit dramatic phenotypic alterations. These alterations included enhanced cytoskeletal organization and FA assembly, as well as changes in cell morphology, which were consistent with an improvement in motility in comparison with the swapped chimeric integrin. This study has identified, at a molecular level, changes in protein-protein interactions that might regulate the assembly of FAs during cell migration, which is a crucial area of cell adhesion research.

Materials and Methods

Generation of SW1 chimeras, GFP fusions of FA proteins and transfection into NIH3T3 cells

Generation of the SW1-swapped integrin chimera has been described elsewhere (Briesewitz et al., 1995). Standard recombinant DNA techniques were used in the construction of the two SβX receptors. Restriction enzymes were obtained from Promega, and Taq DNA polymerase, used for generating DNA fragments, was from Sigma. All constructs were sequenced to confirm their fidelity.

The αx-βcytβc and αx-x10βc subunits (SβX, Fig. 1) were made using as a template the *Bam*HI fragment of pLEN αxβc, including the 3′ region of the α1 extracellular domain and the entire β1 transmembrane and cytoplasmic domains, subcloned into pBluescript.

Stable SβX-expressing NIH3T3 cell lines were created by successive rounds of calcium phosphate co-transfection (initially with pLEN βxαc and subsequently with pLEN SβX) of the expression plasmid (10 μg) with either pSV2neo or pSV2hisD (1 μg). Lines were selected in 1 mg/ml G418 or 3 mM histidinol. Drug-resistant clones were isolated and screened by flow cytometry using anti-human α1 monoclonal antibody (mAb) (TS2/7) (Briesewitz et al., 1995). Multiple positive clones were used in all experiments.

GFP-FAK was prepared as described elsewhere (Ezratty et al., 2005). Murine Cas-GFP was ligated into the pEGFP vector (Clontech) and the stop codon at the 3′ end of the Cas coding region was ablated by site-directed mutagenesis. GFP-Talin was a gift from K. Yamada (National Institutes of Health, Bethesda, MD). Transient transfections of fluorescently labeled FA proteins were performed using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions.

Adhesion-dependent tyrosine phosphorylation of FA proteins

Cells were trypsinized and placed in suspension in Dulbecco's Modified Eagle Medium (DMEM) containing 2% BSA for 1 hour and then plated on coverslips coated with either 1 μg fibronectin or collagen IV (1 μg/ml) for 1 hour. Cells were lyzed in buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM $CaCL_2$, 1 mM $MgCl_2$, 2% glycerol, 10 mM NaF, 1 mM pyrophosphate and 2 mM Na_3VO_4, pH 7.4) supplemented with mammalian extract protease inhibitor cocktail (Sigma). Lysates were used neat (~25 μg) for FAK pY397 determination, or immunoprecipitated with Gammabind G (Amersham) and either anti-Cas (rabbit antibody; Santa Cruz), anti-FAK (mAb 2A7; Upstate) or anti-paxillin (mAb; Transduction) antibodies for 2 hours at 4°C. Equal amounts of protein were electrophoresed in 7.5% gels and transferred to nitrocellulose membranes (GE Osmonics). For FAK phosphotyrosine blots, membranes were probed with a primary polyclonal antibody specific for phospho-Y397 or -Y576 of FAK (Biosource) followed by protein A-peroxidase (Transduction) and subsequently reprobed with anti-FAK (Transduction) or anti-talin (Sigma) antibody followed by anti-mouse-peroxidase (Chemicon). For Cas and paxillin phosphotyrosine blots, membranes were probed with HRP-conjugated anti-pY antibody (RC20; Transduction) and reprobed with Cas (Transduction) or paxillin mAbs. Membranes were developed using enhanced chemiluminescence (ECL; Pierce).

Immunofluorescence microscopy

For immunofluorescence microscopy, cells were plated on collagen IV-coated coverslips in DMEM, 2% BSA, and incubated at 37°C for 60-90 minutes before fixation (PBS, 4% paraformaldehyde, 10 minutes). Cells were stained with Rhodamine-Phalloidin to stain for F-actin or antibodies to either human α1 integrin (TS2/7), vinculin (mAb; Sigma), paxillin-pY118 or FAKpY397 (Biosource). Analysis of Glu-tubulin in cells was performed as described previously (Palazzo et al., 2004). Briefly, sub-confluent cells were trypsinized and placed in suspension in DMEM, 2% BSA with or without 1% serum with constant agitation for 30 minutes. Cells were then plated on collagen IV-coated coverslips for 90 minutes at 37°C before fixation (–20°C methanol, 10 minutes). Cells were then rehydrated in PBS for 5 minutes and stained with a polyclonal antibody for Glu-tubulin and anti-vinculin or TS2/7. All secondary antibodies were from Molecular Probes. Epifluorescence microscopy was performed with a Nikon Optiphot microscope using a 60× plan Apo objective and filter cubes optimized for fluorescein/Alexa Fluor 488 or Rhodamine fluorescence (Nikon). Images were obtained with a MicroMax cooled CCD camera (Kodak KAF 1400 chip; Princeton Instruments) using Metamorph software (Universal Imaging).

Cell migration analysis

For random migration, cells were sparsely plated ($1.0\text{-}1.5\times10^3$ cells/cm^2) onto fibronectin- or collagen IV-coated glass-bottomed culture dishes (MatTek) and allowed to spread for 1 hour. Dishes were then placed on a microscope stage maintained at 35°C and phase images were captured every 20 minutes for 10 hours.

GTPase activation assays

NIH3T3, SβX, SW1 and HA1 cells were grown in 1% serum overnight before being trypsinized and placed in suspension with constant agitation for 1 hour. Cells were plated onto either fibronectin- or collagen IV-coated coverslips in DMEM containing both 1% serum and 2% BSA for the indicated times. Cells were washed with cold PBS prior to lysis (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, 10% glycerol). For Rac activation assays, the plasmid used to prepare the GST-PAK CRIB domain fusion protein was a gift from G. Tarone (University of Torino, Italy). For Rho activation assays, GST-Rhotekin-RBD was obtained from Pierce. Recombinant protein was mixed with glutathione agarose beads for 1 hour prior to incubation with cell lysates (~1 mg for Rac activation and ~10 mg for Rho activation) for 30 minutes at 4°C with constant agitation. Beads were washed with lysis buffer and eluted with SDS sample buffer. Levels of bound GTPase were determined by western blotting using either monoclonal anti-Rac (Transduction) or anti-Rho (Pierce) antibodies. Whole-cell lysates were analyzed to normalize protein levels.

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