An integrin-α4–14-3-3ζ–paxillin ternary complex mediates localised Cdc42 activity and accelerates cell migration

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Summary
α4 integrins are used by leukocytes and neural crest derivatives for adhesion and migration during embryogenesis, immune responses and tumour invasion. The pro-migratory activity of α4 integrin is mediated in part through the direct binding of the cytoplasmic domain to paxillin. Here, using intermolecular FRET and biochemical analyses, we report a novel interaction of the α4 integrin cytoplasmic domain with 14-3-3ζ. This interaction depends on serine phosphorylation of α4 integrin at a site (S978) distinct from that which regulates paxillin binding (S988). Using a combination of metabolic labelling and targeted mass spectrometry by multiple reaction monitoring we demonstrate the low stoichiometry phosphorylation of S978. The interaction between α4 integrin and 14-3-3ζ is enhanced by the direct association between 14-3-3ζ and paxillin, resulting in the formation of a ternary complex that stabilises the recruitment of each component. Although pair-wise interaction between α4 integrin and paxillin is sufficient for normal Rac1 regulation, the integrity of the ternary complex is essential for focused Cdc42 activity at the lamellipodial leading edge and directed cell movement. Taken together, these data identify a key signalling nexus mediating α4 integrin-dependent migration.

Introduction
Integrins are αβ heterodimeric receptors that mediate cell adhesion to the extracellular matrix and enable cell migration. Integrin-ligand engagement induces the assembly of receptor-associated signalling complexes and transduces force between the substratum and the cytoskeleton, thereby controlling cell movement. Although many integrins are ubiquitously expressed, others fulfil specific roles. The α4 integrins are expressed primarily by leukocytes and derivatives of the embryonic neural crest, including melanocytes, and mediate rapid migration and invasion of these cells during development and immune responses (Bochner et al., 1991; Gosslar et al., 1996; Hemler et al., 1990). The α4 integrin subunit pairs with one of two β-subunits, β1 or β7, to form receptors that bind either fibronectin and VCAM-1 (α4β1; VLA-4) (Elices et al., 1990; Mould et al., 1990; Wayner et al., 1989) or MadCAM-1 (α4β7) (Berlin et al., 1993), respectively. As β1 integrin dimerises with a range of α-subunits, the specific properties of α4β1 integrin are determined by the interactions of the α4 subunit. These unique properties are exemplified by the independence of α4β1 integrin from coreceptors during adhesion to fibronectin (Mostafavi-Pour et al., 2003). In contrast to the independent nature of α4β1 integrin, the closely related α5β1 integrin requires synergistic engagement of syndecan 4 for focal adhesion formation on fibronectin (Bass et al., 2007). There is interest in elucidating the unique properties of α4 integrin, as the rapid formation of α4β1-containing adhesions, during leukocyte arrest and migration, is a critical early stage of inflammatory responses, and a validated therapeutic target (Humphries et al., 1994).

Unique among integrin αβ-subunits are α4 and the closely related α9 subunit, in that their cytoplasmic domain can bind directly to paxillin in vitro (Liu et al., 2001; Liu et al., 1999). A dynamic interaction between paxillin and α4 subunit is essential for regulation of motility since disruption of paxillin binding by mutation of an α4 integrin cytoplasmic residue (Y991A), phosphorylation of the cytoplasmic domain at S988 by PKA (Han J. et al., 2001; Liu et al., 1999), or enforced α4 integrin-paxillin association through expression of a fused bimolecular chimera (Han et al., 2003), all lead to increased cell spreading and decreased cell migration. Subsequent studies have shown that α4-paxillin binding is necessary for initial strengthening of adhesion upon cell binding to VCAM-1 under shear stress (Alon et al., 2005) and for leukocyte recruitment to sites of inflammation in vivo (Feral et al., 2006). Clustering of α4 integrin cytodomains has also been found to result in cell motility and Src activation by an unresolved, paxillin-independent mechanism that is indicative of alternative associations of α4β1 integrin (Hsia et al., 2005). In the present study we identify novel interactions of both α4 integrin and paxillin with a 14-3-3 isoform. Formation of this ternary complex facilitates localized Cdc42 regulation and matrix-directed cell migration.

Key words: Integrin, 14-3-3, Paxillin, Cdc42, Migration

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Results

Identification of a putative 14-3-3-binding motif that is specific to the α4 integrin cytoplasmic domain

α4β1 integrin drives focal adhesion formation independently of a coreceptor, such as syndecan 4, or the activation of PKCα (Mostafavi-Pour et al., 2003). This behaviour is distinct from that of α5β1 integrin and is indicative of unique properties of the α4 subunit. To identify domains responsible for the specific properties of α4β1 integrin, a chimeric integrin comprising the α5 extracellular and transmembrane domain and the α4 cytoplasmic domain was compared to full-length integrins for the ability to support focal adhesion formation. Chimeras have been used extensively for analyses of integrin signalling, as they enable the functions of the cytoplasmic domain to be isolated (Chan et al., 1992; LaFlamme et al., 1992). The integrin α-subunits expressed in Chinese hamster ovary cells (CHO-B2), which lack endogenous α5 and α4 integrins, and the cells were spread on the appropriate recombinant ligands of α4β1 or α5β1 integrins. Cells expressing full-length α5 subunit spread on a 50K fragment of fibronectin containing the central cell-binding domain. However, prominent vinculin clusters only formed upon co-engagement of syndecan 4 (Fig. 1A,B), recapitulating the behaviour of fibroblasts adhering to fibronectin through endogenous α5β1 integrin (Bass et al., 2007). Cells expressing the full-length α4 subunit spread on the alternatively spliced IIICS domain of fibronectin (H/120). α4-expressing cells formed vinculin-containing focal adhesions, even when the putative syndecan-4-binding motif of H/120 had been substituted (Fig. 1C), recapitulating the behaviour of melanoma cells adhering to this ligand through endogenous α4β1 integrin (Mostafavi-Pour et al., 2003). Importantly, cells expressing the chimeric integrin behaved like cells adhering through α4 integrin, forming focal adhesions on the integrin ligand without need for additional stimuli (Fig. 1D). This result demonstrates that the unique features of the α4 integrin that promote focal adhesion formation are contained within the cytoplasmic domain.

A direct interaction between the α4 integrin cytoplasmic domain and paxillin has already been characterised and found to inhibit cell migration (Liu et al., 2002). To identify additional α4-binding molecules that might act with paxillin to regulate migration, a bioinformatic analysis of the α4 cytoplasmic domain was conducted using the eukaryotic linear motif resource (Puntervoll et al., 2003). This analysis identified a putative mode II 14-3-3-binding motif, RX(φ)XS(P)XP (where φ designates an aromatic residue, and S(P) is phosphoserine), located between residues R974 and L980 (Fig. 1E). Like the paxillin-binding sequence, this motif is conserved in α4 between species, but is absent from other integrin α-subunits, including α5 (Fig. 1E, and data not shown). The 14-3-3 family comprises seven closely related genes, encoding β−, ε−, η−, τ−, ζ− and σ−isoforms, which function as homo- and heterodimers. All isoforms are highly conserved in mammals (Aitken, 2006) and recognize peptide sequences that include phosphorylated serine or threonine residues (Muslin et al., 1996). Structural studies have shown that, when bound to 14-3-3 dimers, the RX(φ)XS(P)XP motif adopts an extended conformation with a tight turn that includes the cis-proline residue at S(P)+2 (Yaffe et al., 1997). Although this proline is absent from the α4 integrin cytoplasmic tail, secondary structure predictions have proposed a β-turn between 1979 and E983 that could provide a similar structural feature at the equivalent position in the motif (Filardo and Cheres, 1994).

Phosphorylation of serine978 of the α4β1 integrin cytoplasmic domain

Phosphorylation of S978 is a key prerequisite of the hypothetical 14-3-3 binding motif in the α4 integrin cytoplasmic domain. It has already been reported that S988 is phosphorylated by PKA, and it was noted that the S988A substitution did not entirely abrogate serine phosphorylation of the α4 cytoplasmic domain (Han J. et al., 2001). To investigate phosphorylation of S978, wild-type and mutant α5/α4 chimeras were immunoprecipitated from CHO-B2 cells following metabolic labelling with [32P]orthophosphate. Immunoprecipitated α5/α4 resolved into two bands during reducing SDS-PAGE because of the presence of a membrane-proximal cleavage site in the α5 extracellular domain. The 130 kDa band was recognised by an antibody directed against the α5 integrin extracellular domain (H-104), and the 25 kDa band was found by liquid chromatography-tandem mass spectrometry (LC-MS-MS) to include the α4 cytoplasmic domains. Direct comparison of the amount of 32P incorporated into wild-type or S978A α4-cytoplasmic domains confirmed the previously reported reduction in phosphorylation of the S988A mutation, and also revealed residual phosphorylation of this mutant (Fig. 2A) (Han J.

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**Fig. 1.** The cytoplasmic motifs of fibronectin-binding integrin α-subunits. (A–D) CHO-B2 cells expressing α5 and α4 integrin subunits or a chimeric integrin comprising the α5 extracellular domain and the α4 cytoplasmic domain were spread on recombinant fibronectin fragments, appropriate to the extracellular domain, and stained for vinculin (green) and actin (red). Scale bar: 10 μm. (E) Peptide sequence of the cytoplasmic domains of the α4 and α5 integrin subunits indicating the α4 paxillin-binding motif (green) and putative 14-3-3 mode II interaction motif (blue). Paxillin-(Y991A) and 14-3-3- (S978A) binding mutants were generated by site-directed mutagenesis (red).
Method. An ion corresponding to the peptide encompassing phosphorylated S988 was identified with probabilities of greater than 95% by this protocol. Peptides encompassing phosphorylated S988 were intense peaks, which after two occurrences were excluded for 12 spectra were acquired from melanoma cells, phosphorylated peptides were identified by LC-MS-MS. Product ion scans were obtained for peptides with ion transition detected, but the parent ion exhibited the same retention time (11.85 minutes) as the synthetic peptide. The synthetic phosphopeptide was used to obtain accurate empirical measurements of the peptide corresponding to the cytoplasmic domain using H-104, and phosphorylation of the cytoplasmic domain was measured by autoradiography. (C) 2 fmol of synthetic peptide corresponding to the α4 integrin cytoplasmic domain, phosphorylated at S978 was digested with trypsin and added to a 6-protein mix. The mix was infused into the mass spectrometer and the retention spectrum was obtained for the peptide matching the specific neutral phosphate loss transition (534.7/485.7). (D) The light chain of endogenous α4 integrin, immunoprecipitated from A375 melanoma cell, was analysed using the protocol optimised with the synthetic peptide.

Collectively, these experiments show that S978 of the α4 cytoplasmic domain is phosphorylated in both the chimeric and endogenous integrin. This finding demonstrates that the putative 14-3-3-binding motif identified by sequence analysis is indeed present in cells. The experiments also demonstrate that phosphorylation of S978 occurs at lower stoichiometry than phosphorylation of S988, which regulates paxillin binding.

α4 integrin and 14-3-3 closely associate in adhesion contacts

Association between 14-3-3 and the α4 cytoplasmic domain was tested in live cells using acceptor photobleaching fluorescence resonance energy transfer (FRET). The C termini of the chimeric integrins were conjugated to green fluorescent protein (GFP) and coexpressed with 14-3-3ζ to monomeric red fluorescent protein (mRFP). The veracity of the tagged integrin was evident as expression supported cell spreading on 50K and the formation of paxillin-containing focal adhesions, whereas untransfected CHO-B2 cells were incapable of adhering to this ligand (data not shown). The 14-3-3ζ isoform was selected as it has previously been implicated in integrin-mediated signalling (Bialkowski et al., 2003). Transiently transfected cells with intermediate fluorescent intensity were chosen for FRET analysis to avoid non-specific association between the integrin and 14-3-3 as a result of overexpression (supplementary material Fig. S1A,C). FRET was detected in α5/α4-GFP-containing adhesion contacts, demonstrating the close (~10 nm) proximity between the α4 cytoplasmic domain and 14-3-3ζ, and suggesting a direct association (Fig. 3A,D; supplementary material Fig. S2A).

The level of FRET between α5/α4-GFP and mRFP–14-3-3 was 24%, compared with background levels of 10% between mRFP–14-3-3 and free GFP, or between α5/α4-GFP and free mRFP (data not shown). Engagement of a phosphorylated serine residue by the highly conserved amphipathic groove in the 14-3-3 dimer is the key event in 14-3-3 binding, and so we hypothesised that substitution of S978A of α4 would prevent interaction with 14-3-3. No FRET was detected above background between α5/α4-S978A-GFP and mRFP–14-3-3, although both proteins did colocalize at the cell edge, indicating that the mutation abolishes direct association (Fig. 3B,D; supplementary material Fig. S2B).

Phosphomimetic mutation of S978 (S978D), which would be expected to enforce integrin–14-3-3 association, induced widespread FRET between the α4 cytoplasmic domain and 14-3-3ζ (Fig. 3C,D; supplementary material Fig. S2C). The large difference in the FRET of the phosphomimetic, compared with
the wild type, integrin is consistent with low stoichiometry of phosphorylation of the wild-type integrin. FRET analyses were repeated with CHO-B2 cells expressing full-length α4 integrin, to ascertain whether the interaction with 14-3-3 is a feature of the wild-type integrin. FRET between α4-GFP and mRFP–14-3-3 was 33%, comparable with the chimeric integrin (Fig. 4A,D; supplementary material Fig. S3A). FRET was reduced to 16% in the S978A mutant of α4 (Fig. 4B,D; supplementary material Fig. S3B) and more widely spread when enforced by the S978D substitution (34%; Fig. 4C,D; supplementary material Fig. S3C). Taken together, the FRET data reveal an interaction between 14-3-3ζ and the α4 cytoplasmic domain in integrin-containing adhesion contacts that is enhanced by the phosphomimetic substitution of S978.

α4 integrin and 14-3-3ζ form a ternary complex with paxillin

As it has been reported previously that paxillin is essential for α4-mediated cellular responses (Liu et al., 1999), a potential functional link between paxillin and 14-3-3 was explored. Previous investigations have used in vitro binding assays and mutant integrins to establish paxillin binding (Liu et al., 2001; Liu et al., 1999), but the low stoichiometry of the interaction has hampered studies of the endogenous interaction. The association of α4 integrin and paxillin in vivo was verified by measuring FRET between the GFP-tagged integrin chimeras and mRFP-paxillin. The level of FRET between α5/α4-GFP and mRFP-paxillin was only 16% (Fig. 5A) but was reduced to 11% by the Y991A mutation (Fig. 5B), which blocks paxillin binding in vitro (Liu et al., 2002). The difference in FRET efficiency of paxillin, compared with 14-3-3ζ was most

Fig. 3. 14-3-3ζ interacts with the cytoplasmic domain of α4 integrin in adhesion contacts. CHO-B2 cells transiently transfected with (A) α5/α4-GFP, (B) α5/α4-S978A-GFP and (C) α5/α4-S978D-GFP, were co-transfected with mRFP–14-3-3ζ and allowed to adhere to glass-bottomed Petri dishes coated with 10 μg/ml 50K. Spread cells were then subjected to acceptor photobleaching FRET. Line profiles indicate fluorescence intensity emitted by the GFP-conjugated integrin subunits in adhesion contacts before and after mRFP photobleaching. (D) The average peak FRET efficiency of all adhesion contacts per cell. Data are representative of 70-100 adhesion contacts, and the experiment analyzed 10 separate cells per line. Error bars represent s.e.m. *P<0.05. Scale bars: 5 μm.
probably a consequence of the proximity of the fluorophore to the integrin-coupled GFP. Background FRET between mRFP-paxillin and free GFP was 10%, comparable to the Y991A mutant (data not shown). Unlike 14-3-3ζ FRET, in which there was comparable efficiency in small focal complexes and focal adhesions alike, paxillin FRET was more pronounced in established focal adhesions, which supports the existing model that paxillin does not bind to α4 at the leading edge of the cell (Nishiya et al., 2005). This experiment demonstrates that paxillin does indeed bind to α4 integrin in vivo, and does so in focal adhesions.

In its active form, paxillin is phosphorylated at a large number of serine residues (Webb et al., 2005), and it is conceivable that these sites could act as 14-3-3 ligands. A potential interaction between 14-3-3ζ and paxillin was tested by using a 14-3-3ζ-GST pull-down approach to precipitate potential binding partners from a CHO-B2 cell lysate. Analysis of the precipitated proteins by western blotting revealed the previously characterized 14-3-3ζ-binding partners Raf1 (Freed et al., 1994; Irie et al., 1994) and p130Cas (Garcia-Guzman et al., 1999), but also revealed an association with paxillin (Fig. 6A). GST alone did not precipitate any of these proteins. Furthermore, as vinculin and talin were not pulled down by 14-3-3ζ, it is unlikely that multi-molecular complexes of focal adhesion proteins were precipitated. To ensure that interaction occurred at physiological protein concentrations, endogenous 14-3-3ζ was communoprecipitated with paxillin from CHO-K1 lysates (Fig. 6B). Similar association was detected in MEF and 3T3 lysates (data not shown), and α-actinin, which does not interact with paxillin directly, did not co-precipitate with paxillin in these experiments (Fig. 6B). These data therefore reveal a novel association between paxillin and 14-3-3ζ, and raise the possibility that integrin α4, paxillin and 14-3-3ζ form a ternary complex.

To test whether the involvement of paxillin was essential for binding of 14-3-3ζ to α4 integrin, we conducted pull-down assays, using recombinant GST–14-3-3ζ coupled to Sepharose beads. Little wild-type or S978A α5/α4 was pulled down by GST–14-3-3ζ, which was consistent with the low stoichiometry of S978 phosphorylation (Fig. 6C). As expected, substitution of the putative paxillin-binding motif (Y991A), or substitution of both the paxillin and 14-3-3-binding motifs in combination did not improve binding to 14-3-3ζ. Importantly, large amounts of integrin were precipitated from cells expressing α5/α4-S978D/Y991A, demonstrating that α4 integrin can bind to 14-3-3ζ independently of the paxillin-binding motif. The strong association between α5/α4-S978D/Y991A and 14-3-3ζ, compared with wild-type α5/α4 integrin (Fig. 6C), was also consistent with the increased association detected by FRET (Fig. 3C), and the low stoichiometry of phosphorylation of S978 (Fig. 2).

Finally, we tested the complementary roles of 14-3-3ζ and paxillin in ternary complex formation. FRET between α5/α4-GFP and mRFP–14-3-3ζ was reduced from 24% to a background level
of 9% upon substitution of the paxillin-binding motif (Y991A; Fig. 2A, Fig. 7A,B). The effect of Y991A on 14-3-3ζ-binding demonstrates that, in the absence of enforced α4–14-3-3ζ association by S978D, paxillin is important for recruitment of 14-3-3ζ to the integrin. The same result was obtained from the full-length integrin, with FRET reduced from 33% to 8% in cells expressing α4-Y991A-GFP (Fig. 7C,D). The reciprocal relationship was tested by examining the effect of 14-3-3ζ on paxillin recruitment. Upon substitution of the paxillin-binding motif of α5/α4 (Y991A), localisation of paxillin to early focal complexes, but not mature focal adhesions, was compromised (Fig. 7E,F). However, paxillin recruitment was restored by enforcing the association between α5/α4 and 14-3-3ζ (S978D/Y991A; Fig. 7E,F). Recruitment of vinculin was unaffected (Fig. 7G). Collectively these experiments show that although α4 integrin, 14-3-3ζ, and paxillin are capable of forming pair-wise connections, association of endogenous molecules requires formation of a ternary complex. It was interesting that the Y991A substitution only compromised recruitment of paxillin to focal complexes (Fig. 7E, arrowheads) and not to mature focal adhesions (Fig. 7E, arrows). The difference in recruitment patterns suggests that ternary complex formation is specifically important for the formation of nascent focal complexes at a leading edge.

Formation of the α4–14-3-3ζ-paxillin ternary complex is necessary for regulation of Cdc42 during cell migration

The Rho family GTPases, Rac1 and Cdc42, play essential roles in the induction of vinculin- and paxillin-containing focal complexes at the leading edge of migrating cells (Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004), yet despite considerable efforts, the direct link between adhesion receptor and localised GTPase regulation remains poorly understood. Recent studies have revealed that both GTPase regulation and focal complex formation, in response to engagement of the fibronectin receptor α5β1 integrin, require simultaneous engagement of a second matrix receptor, syndecan 4 (Bass et al., 2007). By contrast, integrin α4β1 supported focal adhesion formation that was independent of a second receptor (Mostafavi-Pour et al., 2003), which implies a direct link from the α4 cytoplasmic tail to GTPase regulation. The impact of the α4–14-3-3ζ–paxillin complex on Cdc42 and Rac1 activity was therefore examined by effector pull-down assays. Disruption of the paxillin binding motif of the α4 cytoplasmic tail (α5/α4-Y991A) caused an increase in Rac1 activity (Fig. 8A) that was consistent with previous reports of reduced Rac1 activity upon enforced paxillin-α4 integrin association (Nishiya et al., 2005). However, mutation of the 14-3-3-binding motif (α5/α4-S978A) had no effect on Rac1 activity, and neither did it rescue Rac1 activity in the paxillin-binding mutant (Fig. 8A). By contrast, Cdc42 activity was markedly elevated in cells expressing α5/α4-S978A (Fig. 8B). Cdc42 activity was also slightly increased by α5/α4-Y991A, but could be rescued by enforced association between α4 and 14-3-3 in the α5/α4-S978D/Y991A double mutant. These results demonstrate dysregulation of GTPase signalling upon disruption of binding motifs within the α4 cytoplasmic domain, with paxillin primarily affecting Rac1 and 14-3-3 primarily affecting Cdc42. It is interesting that similar dysregulation of Rac1 is observed in syndecan-4-null cells during α5β1-mediated adhesion (Bass et al., 2007), so that uncontrolled GTPase activity in cases of compromised receptor signalling appears to be a recurring theme.

The role of 14-3-3ζ in localisation of active Cdc42 was examined using the Raichu-Cdc42 FRET probe (Itoh et al., 2002). In cells expressing the wild-type integrin α4 cytoplasmic domain, high FRET efficiency, indicating high Cdc42 activity, was focused along the leading edge of lamellipodia (Fig. 8C,G). By contrast, active Cdc42 was distributed diffusely in cells expressing either α5/α4-Y991A or α5/α4-S978A (Fig. 8D,E,G), confirming the role of the α4–14-3-3ζ–paxillin ternary complex in restricting Cdc42 activity to the leading edge. In the same way that enforced α4–14-3-3ζ association rescued paxillin mislocalisation (Fig. 7E), localised active Cdc42 was restored in the α5/α4-S978D/Y991A double mutant (Fig. 8F,G). In all cases, cells expressing a negative control FRET probe, Raichu-Cdc42Y40C, displayed no FRET activity, demonstrating that the calculated ratios were indicative of activation rather than accumulation of the probe. The FRET data support the concept that 14-3-3ζ, rather than paxillin, exerts the primary influence over α4-dependent Cdc42 regulation, and that the complex restricts active Cdc42 to the leading edge.

The functional relevance of the interaction between integrin α4 and 14-3-3ζ was investigated using a haptotactic cell migration assay. Wild-type CHO-B2 cells were unable to migrate towards the 50K central cell-binding domain fragment of fibronectin as they lack α5β1 expression, but transfection of the full-length α5 subunit stimulated efficient cell migration towards 50K (Fig. 8H). Cell migration was increased by 40% by expression of the α5/α4 chimera compared with expression of α5, indicative of the pro-migratory signalling role of α4 integrins. Abrogation of the association between α4 integrin and 14-3-3ζ by the S978A mutation reduced α5/α4-mediated haptotaxis to a level that was comparable to that of wild-type α5 integrin (Fig. 8H). Thus, interaction between α4
integrin and 14-3-3ζ is a critical event in transduction of the pro-migratory signals of α4 integrin.

Discussion
In this study we identified a novel membrane-linked signalling complex comprising α4 integrin, 14-3-3ζ and paxillin. Initially, we demonstrated pair-wise association between paxillin and 14-3-3ζ by co-immunoprecipitation, α4 integrin and 14-3-3ζ by FRET, and confirmed that the previously reported biochemical association between α4 integrin and paxillin does indeed occur in vivo. Subsequently, we demonstrated that formation of the ternary complex was regulated by the phosphorylation of two independent serine residues within the integrin α4 cytoplasmic tail, and that signalling defects caused by disruption of one of the binding sites could be partially rescued by enforced association at the other. Together, these experiments revealed a close relationship between components of the ternary complex. The α4 integrin–14-3-3ζ–paxillin ternary complex was required for localised Cdc42 activity, whereas association of α4 integrin and paxillin alone appeared sufficient for Rac1 regulation. Finally we found that the association between α4 integrin and 14-3-3ζ made a critical contribution toward rapid haptotactic migration and the formation of nascent focal adhesions. Collectively these experiments describe a new mechanism of αβ1 integrin signalling.

αβ1 integrin has received a great deal of attention as a therapeutic target because of the specific role in targeting leukocyte migration, in particular, from the circulation to the brain. Several pathologies have been linked to defects in αβ1 integrin; polymorphisms in the α4 gene that cause increased expression of the integrin have been linked to susceptibility to multiple sclerosis by augmenting leukocyte migration across the blood brain barrier (O’Doherty et al., 2007), persistent recruitment of leukocytes to the intestine results in Crohn’s disease (Sandborn and Yednock, 2003), and αβ1-mediated adhesion has also been linked to other autoimmune diseases including rheumatoid arthritis and diabetes (Kummer and Ginsberg, 2006). These defects have
prompted the development of a function-blocking anti-α4 antibody, natalizumab, which has been trialled as a treatment of Crohn’s disease and relapsing multiple sclerosis with great success. However, in a limited number of cases, natalizumab treatment resulted in progressive multifocal leukoencephalopathy as a result of increased susceptibility to viral infection (Baker, 2007), and the severity of this side effect highlights why the understanding of integrin α4 signalling and development of more specific drugs is a therapeutic priority. Not all integrin α4β1-related therapy is based upon inhibition of the integrin; tumour-reactive T cells have been targeted to intracranial lesions by expression of α4β1 integrin during the treatment of tumours of the central nervous system (Sasaki et al., 2007). The range of leukocyte-based therapies and in particular the potential side effects make it clear that a detailed understanding of α4β1 integrin function is necessary.

The pro-migratory activity of α4β1 integrin must be explained by unique features of the integrin, which in many respects resembles the ubiquitously expressed α5β1 integrin. This study has focused on the importance of the α4–14-3-3–paxillin complex, and the potential of both 14-3-3ζ and paxillin to act as scaffolds may be important for the creation of a signalling nexus (Turner, 2000; Tzivion et al., 2006). The connection from α4β1 to GTPase signalling, in the absence of a ligand for a second matrix receptor, is of particular interest. The role of syndecan 4 in the regulation of GTPases Rac1 and RhoA downstream of α5β1 integrin is now established (Bass et al., 2008; Bass et al., 2007), and the independence of α4β1 integrin may explain the difference between the two integrins. It is understood that phosphorylation of the α4-paxillin-binding motif, in the protruding lamella, restricts active Rac1 to the leading edge by confining paxillin binding, which in turn blocks Rac1 activation, to the sides and rear of the cell (Nishiya et al., 2005). The inability of 14-3-3 binding to rescue Rac1 activity upon substitution of the paxillin-binding motif suggests that the molecules fulfill divergent as well as mutually supportive roles, which may be important for focal adhesion maturation. 14-3-3 recruitment appears to be a very early event in focal complex nucleation. Phosphorylation of S978 directly influences Cdc42, which is one of the earliest signals during formation of pioneering focal complexes and establishment of cell polarity (Burridge and Wennerberg, 2004;
Raftopoulou and Hall, 2004). The locality, rather than the level of, GTPase activity is the key factor in determining cell polarity and is reflected in this study by the observation that the S978A substitution prevents rapid migration, despite causing high, mislocalised Cdc42 activity. 14-3-3 has already been reported to associate with the leukocyte integrin αLβ2 (LFA-1) and to contribute to the regulation of Rac1 and Cdc42 activity in T cells (Nurmi et al., 2007). The ability of the 14-3-3 family of proteins to associate directly with two leukocyte integrins indicates a possible role in immune cell function, and suggests that this interaction may be a target for therapeutic intervention during inflammatory diseases. 14-3-3ζ has also been implicated in GTPase regulation in platelets (Białkowska et al., 2003). Sequestration of 14-3-3ζ by the transmembrane glycoprotein GP Ib-IX prevented activation of Cdc42 and Rac1 in response to engagement of αIIbβ3 integrin, resulting in compromised cell spreading. Although direct association of 14-3-3 with αIIbβ3 integrin has not been demonstrated, collectively the reports do suggest an active role for 14-3-3 in the GTPase signalling complex.

Of equal note was the identification of a transient interaction between 14-3-3β and the cytoplasmic tails of β1 and β3 integrin subunits during the early stages of cell spreading (Han D. C. et al., 2001). Despite the absence of a consensus phosphoserine residue from the integrin β-tails, 14-3-3β coimmunoprecipitated with the integrin and overexpression increased the rates of cell spreading and migration on fibronectin. The presence of putative 14-3-3-binding motifs in both cytoplasmic domains of αβ1 integrin could make the integrin a high affinity ligand for dimeric 14-3-3. The fact that FRET was lost upon substitution of S987A, despite the β1 tail remaining intact, demonstrated the importance of the α4 motif and could explain why, unlike α5β1 integrin, α4β1 integrin does not require cooperation with a second receptor for the initiation of downstream signalling events.

The presence of 14-3-3-binding motifs within the cytoplasmic domains of both integrin subunits raises certain questions about spatial arrangement, as it is inconceivable that a single 14-3-3 molecule could simultaneously bind α4, β1 and paxillin while at the same time α4 integrin binds both 14-3-3 and paxillin. Based on crystal structures, a generic model of integrin activation involving stages of clustering, unbending, leg separation and conformational rearrangement of the ligand-binding pocket that results in increased ligand affinity has been developed (Luo et al., 2007). Ligand-binding and intramolecular FRET experiments have shown that the model is applicable to α4β1 integrin (Chigaev et al., 2007). One attractive hypothesis is that sequential recruitment of 14-3-3 integrin and paxillin would facilitate reorientation of the integrin legs, allowing the recruitment of other integrin activators, such as talin (Garcia-Alvarez et al., 2003) and causing the gradual development of focal complexes (Fig. 9). The efficiency of FRET between the α4 subunit and 14-3-3ζ in all adhesion complexes, compared with paxillin FRET, which was minimal in focal complexes, supports the model of sequential recruitment. The interaction between α4 and paxillin around the periphery of the leading edge has already been found to prevent off-axial Rac1 activation (Nishiya et al., 2005), and it appears that the α4-14-3-3ζ–paxillin ternary complex might exert a similar influence over Cdc42. If binding of 14-3-3 to α4β1 is indeed an early event in integrin activation one would hypothesise that phosphorylation of S988, regulating paxillin binding, could commit the integrin to either GTPase activation at the leading edge or paxillin binding and GTPase suppression around the periphery of the cell. Resolving the dynamics and recruitment hierarchy of molecules is a difficult challenge, as focal complexes have such a short lifetime themselves, but this is now a priority if we are to understand fully how α4β1 integrin is involved in such efficient cell migration.

Materials and Methods

Materials

Recombinant fibronectin polypeptides encompassing type III repeats 6-10 (50K) and 12-15 including the alternatively spliced IIICS domain (H/H20) were expressed as recombinant polypeptides as described previously (Danen et al., 1995; Sharma et al., 1999). The human 14-3-3ζ construct was a gift from Carol Mackintosh (University of Dundee, UK), the human paxillin construct a gift from Vic Small (IMBA, Vienna, Austria), and the mRFP construct a gift from Roger Y. Tsien (University of California, CA). 14-3-3ζ and paxillin were subcloned using 5’ BamHI and 3’ EcoRI restriction sites into pGEX-4T-1 and mRFPN vectors to generate N-terminal glutathione S-transferase and mRFP tags, respectively. The α5t4d cDNA was constructed by engineering a HindIII restriction site into the α4 cDNA between the transmembrane and cytoplasmic domains, and subsequently subcloning the extracellular/transmembrane domains of the α5 subunit and the cytoplasmic domain of the α4 subunit into the Nol and XbaI restriction sites of pcDNA3.1. Point mutations were introduced into the cytoplasmic domain of the α5t4d chimera by site-directed mutagenesis, and mutant chimeras were then subcloned into the ClaI and PinAI restriction sites of pcDNA3.1/EcoRI by PCR amplification of the integrin cDNA. The following antibodies were used for immunofluorescence and western blotting: hVin-1, mouse anti-human vinculin monoclonal (1:400; Sigma-Aldrich); 349 mouse anti-human αLβ2 monoclonal; IgG and TRITC-conjugated phalloidin from Molecular Probes (Invitrogen, Paisley, Scotland); and Alexa-Fluor-680-conjugated anti-mouse IgG from Jackson (Stratech Scientific, Luton, UK), and Alexa-Fluor-568-conjugated anti-mouse IgG and TRITC-conjugated phallolidin from Molecular Probes (Invitrogen, Paisley, UK). mAb16 rat anti-human α5 integrin monoclonal, for flow cytometry and immunoprecipitation, was a gift from Ken Yamada (NIH, Bethesda, MD).

Cell culture

Chinese hamster ovary B2 (CHO-B2) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with L-glutamine and sodium pyruvate, supplemented with 10% (v/v) foetal calf serum, 10 mM MEM non-essential amino acids, 2 mM L-glutamine and 1% (v/v) penicillin and streptomycin. Transfections were performed on cells at 60-80% confluency using Lipofectamine 2000 (Invitrogen). For FRET analyses, CHO-B2 cells were co-transfected with the various α5t4d-GFP cDNAs, mRFP-14-3-3ζ, and mRFP-paxillin were used 24 hours post-transfection, without cell sorting. For biochemical and immunofluorescence analyses, homogenous integrin expression between cell lines, transfected with the various α5t4d integrin cDNAs, was achieved by fluorescence-activated sorting of cells labelled with mAb16 and a FITC-conjugated secondary antibody, using an Aria flow cytometer (BD Biosciences). Sorted populations were maintained in 0.2 mg/ml G418 (Calbiochem). Expression levels of both transiently transfected and sorted cells were assessed using
a Cyan flow cytometer (Dako). A375 human melanoma cells, which express endogenous αβ1 integrin, were cultured in DMEM supplemented with L-glutamine and 10% (v/v) fetal calf serum.

**Immunfluorescence**
Glass coverslips, derivatized with 1 mM sulfho-m-maleimidobenzoyl-N-hydroxysuccinimide ester, were coated with 10 μg/ml 50K or H/120 for 1 hour and blocked with 10 mg/ml heat-denatured bovine serum albumin. CHO-B2 cells (5×10⁴) in DMEM with 25 mM Hepes were added to the coverslips, and the cells were incubated for 90 minutes at 37°C. Spread cells were fixed with 4% (v/v) paraformaldehyde, and permeabilised with 0.5% (v/v) Triton X-100 in phosphate-buffered saline (PBS), and blocked with 3% (w/v) BSA in PBS. Fixed cells were stained for vinculin, paxillin and actin, and photographed using a DeltaVisionRF microscope (Applied Precision, Issaquah, WA) with a 60× NA 1.42 Planapo objective and Photometrics CH350 camera microscope.

**Metabolic labelling**
CHO-B2 cells expressing c5/α–δ-S988A, or c5/α–ζ-S978A/S988A were sorted using 1 μg mAb16 and anti IgG-conjugated magnetic beads (Dynal) to achieve homogeneous integrin expression across the population. At 70% confluence, cells were moved into serum-free, phosphate-free medium (Invitrogen) for 1 hour to allow

\[ \frac{5}{10^4} \]

μ (534.7/485.7) in DMEM with 25 mM Hepes were added to the coverslips, and the dish was blocked with 10 mg/ml heat-denatured bovine serum albumin. CHO-B2 cells

\[ \frac{5}{10^4} \]

μ (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 5 mM MgCl₂, 5 mM EGTA, 0.5 mM AEBSF [4-2-aminoethyl] benzene sulfonyl fluoride), 25 μg/ml aprotinin, 25 μg/ml leupeptin, 5 mM Na₂VO₄, 10 mM NaF, 1× phosphatase inhibitor cocktail 1 (Sigma-Aldrich). Clarified lysates were pre-cleared for 30 minutes with protein G-Sepharose (Zymed) and non-immune rat IgG, before immunoprecipitating the chimeric integrins using 1 μg mAb16 monoclonal antibody and protein G-Sepharose. Proteins were solubilised in SDS sample buffer and resolved by reducing SDS-PAGE. Dried PAGE gels were exposed to a phosphorimager screen overnight, and scanned using a Bio-Rad, Molecular Imager FX.

**Phosphorylation analysis by mass spectrometry**
A synthetic peptide corresponding to the c4 cytoplasmic, phosphorylated on S978 (GFKRQYKJ/P)IQLEENRDDSWSYINSKND) was purchased from Genosys (Sigma).

**Infusion/optimization**
For optimization studies, the peptide was infused into the mass spectrometer using a syringe pump. The optimum collision energy needed to obtain the neutral loss transition for the phosphopeptide of interest (534.7/485.7) was determined. For optimization studies, the peptide was infused into the mass spectrometer using a syringe pump. The optimum collision energy needed to obtain the neutral loss transition for the phosphopeptide of interest (534.7/485.7) was determined.

**Immunoprecipitation**
A375 melanoma cells, spread on H/120 for 60 minutes were lysed in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 5 mM EGTA, 0.5 mM AEBSF, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 5 mM Na₂VO₄, 10 mM NaF, 1× phosphatase inhibitor cocktail 1 (Sigma-Aldrich). Clarified lysates were pre-cleared for 30 minutes with protein G-Sepharose (Zymed) and non-immune rat IgG, before immunoprecipitating the chimeric integrins using 1 μg mAb16 monoclonal antibody and protein G-Sepharose. Proteins were solubilised in SDS sample buffer and resolved by reducing SDS-PAGE. Dried PAGE gels were exposed to a phosphorimager screen overnight, and scanned using a Bio-Rad, Molecular Imager FX.

**Quantitative Western blotting**
Transferred proteins were detected using the Odyssey western blotting fluorescent detection system (LI-COR Biosciences UK, Cambridge, UK). This involved blocking the membranes with blocking buffer (LI-COR Biosciences UK, Cambridge, UK) and then incubating with the primary antibodies diluted 1:1000 in blocking buffer, 0.1% (v/v) Tween 20. Membranes were washed with PBS, 0.1% (v/v) Tween 20 and incubated with Alexa-Fluor-680-conjugated anti-mouse IgG diluted 1:5000 in blocking buffer, 0.1% (v/v) Tween 20. After rinsing the membranes, proteins were detected using an infrared imaging system that allowed both an image of the membrane and an accurate count of bound protein to be recorded. For all experiments, equivalent loading between cell lines was confirmed by blotting the crude lysate for total GTPase. For quantification, values for active GTPase were adjusted for the slight variations in total GTPase. Paired two-tailed t-tests were used for statistical analysis.

**Haptotactic migration assay**
Migration assays were performed using Transwell chambers (Costar). The lower surface of the membrane was coated with 10 μg/ml 50K in PBS containing calcium and magnesium for 2 hours at 37°C. Both the top and the bottom chambers were then blocked with 0.5% (w/v) BSA in serum-free medium for 30 minutes at room temperature. After washing, 1×10⁵ CHO-B2 cells were added to the top chamber and allowed to migrate at 37°C for 4 hours. The cells were subsequently trypsinised from the upper and lower chambers and counted using a Coulter counter. Two-tailed t-tests were used for statistical analysis.

**Raichu-Cdc42 FRET**
The Raichu-Cdc42 and Raichu-Cdc42Y40C FRET probes, a gift from Michiyuki Matsuda (Research Institute for Microbial Diseases, Osaka University, Japan) (Itoh et al., 2002) were transfected into CHO-B2 cells using Lipofectamine 2000. Following transfection, 28 hours later, cells were allowed to spread on 50K-coated 35-mm glass-bottomed, poly-L-lysine-coated, γ-irradiated Petri dishes coated with 10 μg/ml 50K. Cells were fixed with 4% (w/v) paraformaldehyde in PBS, washed and paraformaldehyde quenched with 0.1 M glycine in PBS for 20 minutes. Fixed cells were photographed in the GFP channel on a DeltaVisionRT microscope using a 100× NA 1.35 UPlanapo objective and Photometrics CH350 camera at 2×2 binning. To calculate FRET efficiencies, a GFP image (donor) was captured before and after photobleaching the mRFP channel (acquired in the RFP channel). Following background subtraction, percentage FRET efficiency was calculated, on a pixel by pixel basis, as 100×1/(donor intensity after photobleaching/donor intensity after photobleaching), using ImageJ software (Wayne Rasband, NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). FRET images were displayed as a colour intensity scale. Percentage FRET efficiency values represent the average peak FRET efficiency of 100±150 adhesion contacts, defined by integrin clustering, of each individual cell. Two-tailed t-tests were used for statistical analysis.

**GST pull-down assay and immunoprecipitation**
In vitro association of GST–14-3-3ζ with c5/α4 mutants or pavillin was assayed by GST pull-down. CHO-B2 cells were resuspended to 6×10⁶ cells/ml in lysis buffer (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100, 2 mM AEBSF, 5 mg/ml aprotinin, 5 mg/ml leupeptin, 2 mM sodium vanadate, 10 mM NaF) and incubated at 4°C for 10 minutes. The lysates were cleared and the supernatant incubated with GST or GST–14-3-3ζ beads for 2 hours at 4°C. For communoprecipitation studies, CHO-K1 cells were lysed as above and the supernatant cleared with protein G-Sepharose beads and 1 μg/ml mouse IgG at 4°C. The supernatant was incubated with mouse IgG- or anti-pavillin-coated beads overnight at 4°C. For both methods, the beads were washed, and bound protein were resolved by SDS-PAGE and transferred to nitrocellulose.

**Rac1 and Cdc42 effector pull-down assay**
Cells were allowed to adhere to 50K or kept in suspension, and lysed in 20 mM Hepes, pH 7.4, 10% (v/v) glycerol, 140 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 4 mM EGTA, 1 mM EDTA, 1 mM AEBSF, 50 μg/ml aprotinin, 100 μg/ml leupeptin. Clarified lysates were incubated for 1 hour at 4°C with GST- PAK CRIB domain beads, before washing the beads with lysis buffer. Active GTPase was eluted in SDS sample buffer, resolved by SDS-PAGE and transferred to nitrocellulose.

**Quantitative Western blotting**
Transferred proteins were detected using the Odyssey western blotting fluorescent detection system (LI-COR Biosciences UK, Cambridge, UK). This involved blocking the membranes with blocking buffer (LI-COR Biosciences UK, Cambridge, UK) and then incubating with the primary antibodies diluted 1:1000 in blocking buffer, 0.1% (v/v) Tween 20. Membranes were washed with PBS, 0.1% (v/v) Tween 20 and incubated with Alexa-Fluor-680-conjugated anti-mouse IgG diluted 1:5000 in blocking buffer, 0.1% (v/v) Tween 20. After rinsing the membranes, proteins were detected using an infrared imaging system that allowed both an image of the membrane and an accurate count of bound protein to be recorded. For all experiments, equivalent loading between cell lines was confirmed by blotting the crude lysate for total GTPase. For quantification, values for active GTPase were adjusted for the slight variations in total GTPase. Paired two-tailed t-tests were used on the same gel were used for statistical analysis.

**Integrin α4–14-3-3ζ–pavillin complex 1663**
produced of CFP:YFP to CFP:CFP to give the ratio of YFP to CFP emitted. Images were then displayed in a colour intensity scale. Image processing was performed using the Imaged software.

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