Multiple factors contribute to integrin-talin interactions in vivo

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Summary

The cytoplasmic protein talin is an essential part of the integrin-cytoskeleton link. We characterized the interaction between integrin and two conserved regions of talin, the N-terminal 'head' domain and the C-terminus, which includes the I/LWEQ domain, within the living organism. Green-fluorescent-protein-tagged head and C-terminal domains were recruited to integrin adhesion sites. Both required integrins for recruitment, but the C-terminal domain also required endogenous talin, showing it was not recruited directly by integrins. We used chimeric transmembrane proteins containing the cytoplasmic domain of the integrin β subunit to examine the integrin-talin head interaction. Monomeric chimeric proteins did not recruit talin head, whereas dimeric chimeras efficiently recruited it and caused a strong inhibition of integrin-mediated adhesion. These chimeras recruited surprisingly few integrin-associated proteins, indicating that recruitment of talin did not initiate a cascade of recruitment. Mutagenesis of the integrin cytoplasmic domain, within the chimera, showed the dominant-negative inhibition was not due to talin sequestration alone and that additional interactions are required.

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Introduction

Cell adhesion to the extracellular matrix (ECM) plays an important role in many biological processes. A surprisingly large variety of proteins are associated with sites of cell adhesion to the ECM, including ECM ligands, transmembrane adhesion receptors, scaffolding or adapter proteins, cytoskeleton-binding proteins and signalling proteins. Thus, the molecular mechanism of cell adhesion to the ECM has proved to be much more complex than anticipated.

Cell adhesion to the ECM is primarily mediated by the integrin family of transmembrane adhesion receptors, each composed of an αβ heterodimer. Integrin adhesion requires a link between the cytoplasmic domain of the integrin β subunit and the cytoskeleton, and many proteins have been identified that may contribute to this link (reviewed by Liu et al., 2000). A genetic approach in *Drosophila melanogaster* has revealed that several genes sharing a mutant phenotype with integrin genes encode orthologues of proteins identified in mammalian cells as part of this link, including integrin-linked-kinase (ILK), PINCH, talin and tensin (Brown et al., 2002; Clark et al., 2003; Torgler et al., 2004; Zervas et al., 2001). Comparison of phenotypes caused by the complete absence of each integrin-associated protein revealed how crucial they are for the diverse functions of integrins. Talin stands out in this analysis, as its absence caused almost identical defects to the absence of integrins, in contrast to the others, whose absence caused only a subset of the defects. This indicated that talin is essential for most of functions of integrins, and suggests that integrins and talin form a core complex at the heart of the large complex of proteins associated with integrin adhesive sites. This fit very well with research on integrin-talin interactions in mammalian cells.

Talin was the first cytoplasmic protein identified that binds to integrins (Horwitz et al., 1986), but this interaction has proven to be complex. Talin is approximately 2500 amino acids long and is composed of a small (50 kDa) head domain and a larger (200 kDa) rod domain; the two domains are separated by a calpain proteolytic cleavage site. The head domain is a FERM (band 4.1, ezrin, radixin, moesin) domain, which in turn is made up of three subdomains (F1, F2 and F3); F3 has the same structural fold as phosphotyrosine binding (PTB) domains, which bind to NPxY motifs (Calderwood et al., 2002; Pearson et al., 2000). The cytoplasmic tails of most integrin β subunits contain two well-conserved NPxY motifs within their approximately 47 residue length, and it is the β subunit within the integrin αβ heterodimer that forms the primary link to the cytoskeleton. In vitro, the talin head domain binds directly to the first NPxY motif when nonphosphorylated, as confirmed by X-ray crystallography and NMR (e.g. Garcia-Alvarez et al., 2003). Thus, talin conforms to the function of other proteins containing a FERM domain by binding transmembrane proteins (Bretscher et al., 2002). The second domain of talin shared with other proteins is the I/LWEQ domain (McCann and Craig, 1999). This is an actin-binding domain that is essential for talin function in mammalian cells (Jiang et al., 2003). The relative importance of these different domains is not yet clear, and whereas current results strongly suggest that talin binds directly to the integrin

cytoplasmic tail within the cell and connects it to actin, this has yet to be fully demonstrated.

Recent results in mammalian cells support the view that the interaction between talin and integrin is an early event in the assembly of the integrin-cytoskeleton link. Talin is necessary for the initial formation of the link, as measured by the formation of a 2pN bond (Jiang et al., 2003). This ability of talin requires the I/LWEQ domain, possibly through its actin-binding activity. In addition, talin has a second role reinforcing the integrin-cytoskeletal link, possibly by recruiting other proteins, such as paxillin, vinculin and tensin (Giannone et al., 2003; Torgler et al., 2004). Talin was not required for adhesion-dependent phosphorylation of focal adhesion kinase (FAK) or Src family members. Talin, therefore, is crucial for the physical link between integrins and the cytoskeleton, but not integrin signalling (Brown et al., 2002; Giannone et al., 2003). Talin has also been found to be necessary and sufficient for the inside-out activation of integrins, in cases where the affinity of integrins for soluble ligand is regulated by the cell (Tadokoro et al., 2003).

We have used the molecular genetic tools of *Drosophila* to address three aspects of talin function. First, we gained evidence that, within the intact organism, the talin head interacts directly with the integrin cytoplasmic tail. Second, we examined what features of the integrin cytoplasmic tail are required for the interaction, including the level of oligomerization and the role of specific primary sequence motifs. Third, we addressed the question of whether it is the sequestration of limiting amounts of active talin that accounts for the dominant negative activity of chimeric transmembrane proteins containing the cytoplasmic tail of the integrin β subunit. A variety of such chimeras have been generated, including fusions to the interleukin 2 receptor, CD2, CD4, cadherin, and the *Drosophila* receptor tyrosine kinase Torso (LaFlamme et al., 1994; Lukashev et al., 1994; Martin-Bermudo and Brown, 1996; Martin-Bermudo and Brown, 1999; Smilenov et al., 1994). They can be constitutively active for signalling, in assays such as FAK phosphorylation and regulation of gene expression. In addition, they act as dominant negatives, inhibiting endogenous integrin function. Two models to explain the dominant negative effect are: (1) a feedback model, where excessive signalling shuts off the endogenous integrins and, (2) a competition model, where the integrin cytoplasmic domains of the chimeras sequester limiting amounts of cytoplasmic proteins required for endogenous integrin function. Talin is a good candidate for the target of such competition, because it is required for the initial events of integrin function and because – if talin needs to be activated, as recent work suggests (Martel et al., 2001) – then the amount of active talin could be limiting. Moreover, recent work has shown that competition for talin underlies 'trans dominant' inhibition, where ligand binding and subsequent sequestration of talin by one integrin inhibited the function of a different integrin (Calderwood et al., 2004).

In our examination of integrin-talin interactions within the developing embryo, we concentrated on the major site of integrin adhesion in the embryo: the muscle attachment sites (Bokel and Brown, 2002). Integrins are essential for the linkage that transfers the force of muscle contraction to the exoskeleton. Each end of each muscle attaches via integrins and the extracellular matrix to specialised epidermal cells called tendon cells (for diagram see supplementary material Fig. S1). Integrins and associated intracellular proteins become highly concentrated at the muscle attachment sites, and in their absence the muscles detach and round up shortly after muscle contraction begins.

Our analysis has supported the model that the talin head binds directly to the cytoplasmic tail of integrin, but produced unexpected results regarding the requirements for the integrin cytoplasmic tail in the recruitment of talin and other integrin-associated proteins.

Results

Diverse mechanisms of recruitment of talin N-terminal and C-terminal domains.

To examine whether the talin head domain interacts with integrins in vivo, we generated a construct encoding the talin head domain fused to green fluorescent protein (talinH-GFP), under the regulation of the inducible tissue-specific GAL4-UAS system (Brand and Perrimon, 1993). This allowed analysis of binding between the talin head and integrins that is independent of any additional interactions made by other regions of talin. We also generated a similar fusion with the C-terminal region of talin (GFP-talinC), which includes the actin-binding I/LWEQ domain (McCann and Craig, 1999). The two fusion proteins were expressed in embryonic muscles, which produce the major integrin-containing adhesive junctions in the embryo: the muscle attachment sites (see supplementary material Fig. S1).

Both talinH-GFP and GFP-talinC were recruited to these sites of integrin adhesion, which contain endogenous talin (Fig. 1). Each fusion protein also had additional sites of distribution in the cell, distinct from talin. TalinH-GFP had significant diffuse cytoplasmic staining, stronger than that of talin but, more surprisingly, it also accumulated in the nuclei (Fig. 1C). The talinH-GFP construct includes the putative calpain cleavage site in talin, raising the concern that the high cytoplasmic and nuclear fluorescence was due to GFP that had been cleaved from the talin head. However, western blot analysis with anti-GFP antisera showed that the fusion protein was not cleaved (Fig. 1H). To confirm that the complete fusion protein was within the nucleus, we raised an antibody against the talin head domain. In wild-type embryos this antibody strongly stained the muscle attachment sites, identical to the antibodies raised against the talin C-terminus (Fig. 1, compare I with A). In embryos expressing talinH-GFP the antibody against the talin head showed the same distribution as that revealed by the GFP tag, with strong staining in the cytoplasm and nucleus in addition the muscle attachments (Fig. 1, compare J with C), demonstrating that intact talinH-GFP is concentrated in the nucleus. This distribution is similar to that seen when fluorescently labelled talin fragments that include the talin head domain were injected into fibroblasts; they not only localized to focal adhesions but were also distributed diffusely in the cytoplasm and the nucleus (Nuckolls et al., 1990). GFP-talinC was found in bright cytoplasmic puncta, which appear to be precursors of the Z-lines because at later stages GFP-talinC accumulated at Z-lines (Fig. 1D). Thus, both the N-terminal head of talin and the C-terminal I/LWEQ containing domain are recruited to sites of integrin function, although not as tightly as full-length talin, either when expressed at endogenous levels or when overexpressed (Fig. 1A,B).
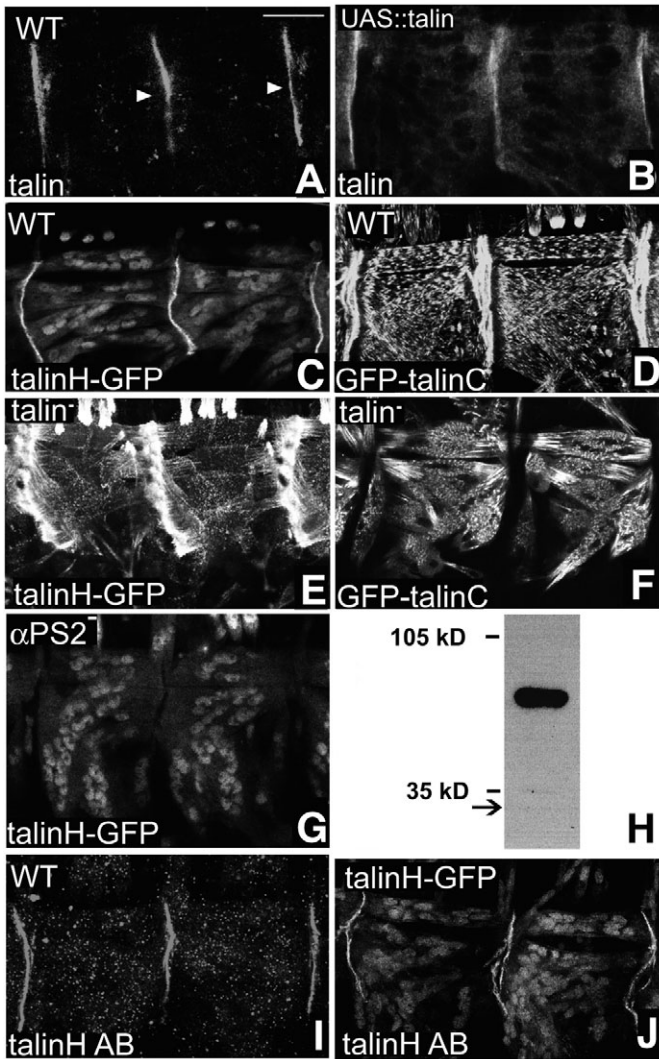
Fig. 1. Localisation of talin N- and C-terminal domains in the embryo. (A-G) Confocal images of muscles from two segments of a late-stage *Drosophila* embryo. A,B are anti-talin stained fixed embryos and C-G are images of GFP fluorescence in live embryos. (A) Talin is enriched at integrin adhesion sites at muscle ends (arrowheads). (B) Elevating talin expression with *mef2::Gal4* and *UAS::talin* resulted in a modest increase in localized talin and a larger increase in cytoplasmic talin. The *mef2::Gal4* driver was also used to express the following domain fusion proteins. TalinH-GFP (C) was localized to muscle ends, but expression in the cytoplasm and nuclei was also observed. GFP-talinC (D) localized to muscle ends and the developing Z-lines. (E,F) Reduction in endogenous talin caused an increase in talinH-GFP recruitment to muscle ends and a loss of GFP-talinC. (G) Removal of αPS2βPS integrin caused the loss of talinH-GFP from the muscle ends. (H) TalinH-GFP, detected with an anti-GFP antibody, remained intact despite the putative calpain cleavage site between talinH and GFP. Arrow indicates where cleaved GFP would migrate. (I) An antibody raised against the talin head domain stains muscle attachments in wild-type embryos. (J) embryos expressing talinH-GFP also showed nuclear and cytoplasmic staining, identical to the GFP fluorescence from this fusion protein (C). Bar, 20 μm.

We expected that overexpression of these individual domains of talin might lead to defects in integrin function. Overexpression of the talin head in mammalian cells leads to strong inside-out activation of integrins (Calderwood et al., 1999). In addition, it would have been possible that expression of just one interaction domain produces a dominant negative effect by competing for endogenous talin and disrupting its linking function. However, we did not observe any defects due to the expression of either construct. In particular, the overexpressed head domain did not cause the expansion of muscle attachment sites previously observed when constitutively active integrins were expressed (Martin-Bermudo et al., 1998). The expression of the domains did not grossly alter the recruitment of endogenous talin in muscles or give rise to a phenotype in other tissues where integrin function is known to be important (data not shown). This suggests that endogenous talin is more effective at binding to integrins or other target proteins than the individual domains, and thus resistant to potential dominant negative effects.

To test competition between the individual domains and endogenous full-length talin, we analysed the effect of reducing the amount of endogenous talin on the localisation of the two domains. The pool of talin in the embryo is a combination of maternally deposited protein and zygotically expressed protein. In the absence of zygotic talin expression the amount of talin at the muscle attachment sites in the late embryo is substantially reduced (Brown et al., 2002). Reducing endogenous talin in this way resulted in an increase of talinH-GFP at muscle ends, with a corresponding decrease of cytoplasmic and nuclear fluorescence (Fig. 1E). This confirmed that talin competes with talinH-GFP for available recruitment sites at muscle ends, and showed that there is nonlocalised excess talinH-GFP in the cytoplasm and nucleus. In comparison, GFP-talinC was no longer recruited when talin was reduced and instead decorated actin filaments (Fig. 1F). Therefore, the recruitment of GFP-talinC to the muscle ends was through interaction with talin or a protein recruited by talin. A fragment of talin containing this domain dimerizes in vitro (Senetar et al., 2004), suggesting direct recruitment of GFP-talinC by endogenous talin. However, GFP-talinC distribution does not exactly mimic talin, which is not at high levels in the Z-lines, suggesting that talinC interacts with other proteins. Furthermore, the individual domains still did not cause a dominant negative effect when endogenous talin was reduced, nor were they able to rescue the mutant phenotype caused by talin reduction.

Talin recruitment to muscle attachment sites requires the sole integrin heterodimer known to be expressed in the muscles, αPS2βPS, because recruitment is abolished in the absence of βPS or αPS2 (Brown et al., 2002). Similarly, recruitment of talinH-GFP and GFP-talinC to muscle attachments was lost when βPS or αPS2 was removed from the embryo. TalinH-GFP instead accumulated in the cytoplasm and nuclei (data not shown and Fig. 1G), whereas GFP-talinC localized with actin fibres (data not shown), as was seen when talin was reduced (Fig. 1F).

In summary, both talinH-GFP and GFP-talinC were recruited to sites of integrin-mediated adhesion at the muscle attachment sites, but their mechanism of recruitment differs. The head domain required integrins and competed with endogenous talin for limited binding sites, fully consistent with the talin head binding directly to the integrins. For the talin C-terminal domain, its recruitment to muscle ends required both

integrins and endogenous talin, and only when these molecules were reduced did GFP-talinC associate with actin filaments. This suggests that the primary binding partner for the recruitment of the C-terminal part of talin in this assay were neither integrins nor actin, but instead is another protein associated with integrin adhesion sites.

Integrin recruitment of talinH-GFP

Additional experiments were performed with the goal of discovering whether the recruitment of talinH-GFP occurred by direct binding to the β integrin cytoplasmic tail or indirectly through other intermediate proteins. We first tested whether elevation of integrin levels cause a corresponding increase in recruitment of talinH-GFP. We overexpressed both αPS2 and βPS integrin subunits. Perhaps most significantly, we found that these overexpressed integrins could recruit talinH-GFP in myoblasts – as seen by a shift of talinH-GFP from nucleus and cytoplasm to the cell cortex (Fig. 2A-C). Myoblasts do not normally form prominent integrin junctions and, therefore, might not contain the full complement of proteins found later at integrin adhesive junctions. For example, GFP-talinC was not recruited in a similar manner (data not shown), indicating that the intermediary protein required for its recruitment is not present or active.
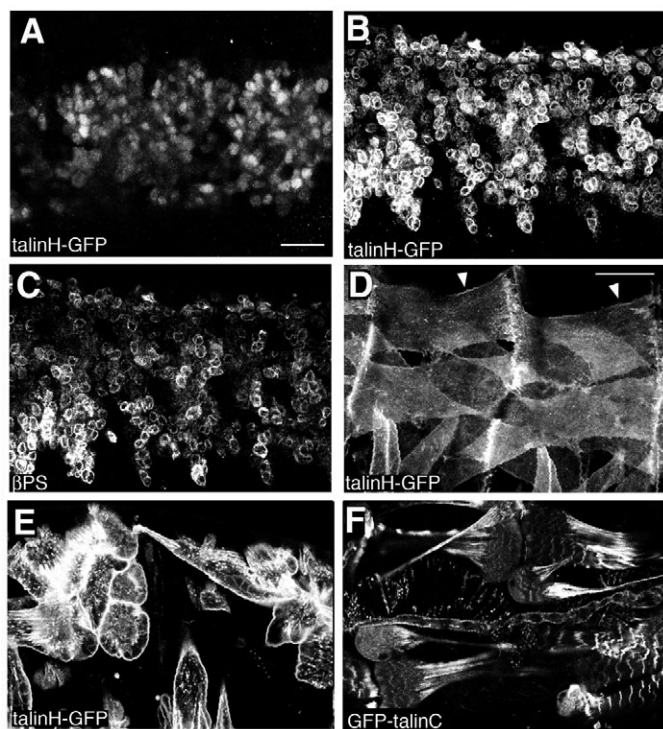

Fig. 2. Elevated levels of integrin increased talin head recruitment. (A-F) Developing muscles at different stages. (A) Wild type; (B-F) elevated integrin expression (*UAS::βPS/+; UAS::αPS2/24BGal4*). (A) In wild-type myoblasts, integrin levels are too low to recruit talinH-GFP, which was found in the nucleus. Overexpressed integrins (C) recruited talinH-GFP (B) to the cortex. In fully fused muscles overexpressed integrin recruited talinH-GFP (arrowheads) out of the nucleus and to the ends (D) (compare with Fig. 1C). Later on, the elevated integrin caused muscle detachment and talinH-GFP was recruited to the cortex (E), in contrast to GFP-talinC (F). Bars, 20 μm.

We also saw that talinH-GFP could be recruited by overexpressed αPS2βPS in both normal and ectopic locations in mature muscles. Increased levels of talinH-GFP were found at the ends of muscles and, more easily visible, its distribution in cytoplasm and nuclei was reduced (Fig. 2D), similar to when we reduced endogenous talin. In addition, talinH-GFP was recruited to an abnormal position on the lateral sides of the muscles (Fig. 2D, arrowheads). The αPS2βPS overexpression caused a dominant negative muscle detachment phenotype that is characteristic of the absence of integrin function. This appeared to be caused by an excess of the βPS subunit; overexpression of βPS alone caused a much more severe phenotype, and the use of a different line of the *UAS::βPS* transgene that produced lower levels of protein in combination with *UAS::αPS2* did not cause the same defects (data not shown). In muscles detached by excess integrin subunits, talinH-GFP was strongly recruited to the cortex (Fig. 2E). GFP-talinC was not recruited to the cortex but instead remained at the remnants of the normal attachment sites and associated with actin filaments (Fig. 2F). Thus, these experiments have provided two examples where excess integrins produced an abnormal cortical distribution of talinH-GFP but failed to recruit GFP-talinC, supporting the model that the talin head binds integrins directly.

Recruitment of talinH-GFP by chimeric transmembrane proteins containing the integrin cytoplasmic tail

We sought a method to further reduce the number of proteins associated with the cytoplasmic domain of integrins to gain additional support for a direct interaction between talinH-GFP and integrins. The generation of mechanical tension in the integrin-cytoskeletal linkage contributes to the assembly of focal adhesions (Galbraith et al., 2002). By using chimeric transmembrane proteins containing extracellular domains that do not mediate adhesion in *Drosophila* fused to the βPS cytoplasmic tail, we should eliminate the ability of the integrin to resist mechanical force. We first used monomeric chimeras of mammalian CD2 and βPS, containing the transmembrane domain from either CD2 (CCβ) or βPS (Cββ) (Martin-Bermudo and Brown, 1996). The presence of endogenous integrin made it difficult to assess elevation of talinH-GFP at muscle ends, so depletion in the cytoplasm and nuclei was assayed. Neither CCβ nor Cββ recruited talinH-GFP from the cytoplasm and nucleus to muscle ends, although both were expressed and localized to muscle attachments (Fig. 3A; data not shown). To better analyze recruitment to the cell cortex, we measured the average intensity of the GFP signal in cell cortex and cytoplasm and calculated the ratio of cortical to cytoplasmic signal. When talinH-GFP is expressed in wild type, the ratio of cortical to cytoplasmic staining is 0.90±0.08 (Fig. 3C). Overexpression of αPS2βPS resulted in an increase of about 40% in the ratio indicating a substantial increase in the amount of staining at the cell cortex. In comparison, expression of either CCβ or Cββ did not alter the ratio, which shows that, in this context, the cytoplasmic domain of βPS was not sufficient to recruit the talin head.

We previously showed that a monomeric chimera was not able to mimic integrin signalling (as assayed by regulation of gene expression in the embryo), whereas a dimeric and/or oligomeric chimera was able to send constitutive integrin signals (Martin-Bermudo and Brown, 1999). This dimerized

chimera (diβ) was made from the extracellular and the transmembrane domain of a constitutively-active mutant form of the *Drosophila* receptor tyrosine kinase Torso, which is thought to dimerize or oligomerize independently of ligand. In addition to being constitutively active for integrin signalling (Martin-Bermudo and Brown, 1999), diβ also acts as a dominant negative protein for integrin adhesion (M.D.M.-B., N.H.B., unpublished observations) (Narasimha and Brown, 2004), an observation that was not found with the CD2βPS chimeras. As outlined in the introduction, the dominant negative activity of diβ could arise from excessive signalling or competition. Since this construct was more biologically active than the CD2βPS chimeras, we tested whether it was able to recruit talin head. Expression of diβ resulted in increased talinH-GFP at muscle termini, reduced levels in the nuclei and cytoplasm, and ectopic recruitment to the cell cortex at sites other than muscle termini (Fig. 3B). We quantified the ratio of cortical to cytoplasmic staining in muscles that expressed diβ and found an increase of about 40% similar to the increase that was seen when αPS2βPS was overexpressed and indicating a large increase in cortical staining (Fig. 3C). We also tested a second diβ chimera (diβY) made with a weaker constitutively-active mutant form of Torso, which was thought to be weaker owing to less ligand-independent dimerization and/or oligomerization (the Y9 allele versus the 4021 allele) (Dickson et al., 1992; Sprenger and Nusslein-Volhard, 1992). We found that expression of diβY induced a milder dominant negative phenotype and that recruitment of talinH-GFP was reduced compared with the original diβ construct (supplementary material Fig. S2). We can, therefore, correlate the levels of dimerizing activity of the diβ construct with its ability to recruit talinH-GFP. Thus, the ability of chimeric transmembrane proteins containing the βPS cytoplasmic domain to recruit talinH-GFP is correlated with the degree to which they form dimers or oligomers, suggesting that dimerization or clustering of integrins is a prerequisite for interaction with talin in vivo. Since this finding contrasts with in vitro analysis of the interaction (Pfaff et al., 1998), it remains possible that some feature of the TorsoβPS fusion (besides its ability to dimerize) promotes the ability of the βPS cytoplasmic domain to recruit talin, but dimerization is the simplest explanation.

We next examined how many of the proteins normally associated with integrin-adhesive junctions can be recruited to the cell cortex of the muscles by diβ. In *Drosophila*, the proteins found to associate with integrins include ILK, PINCH,
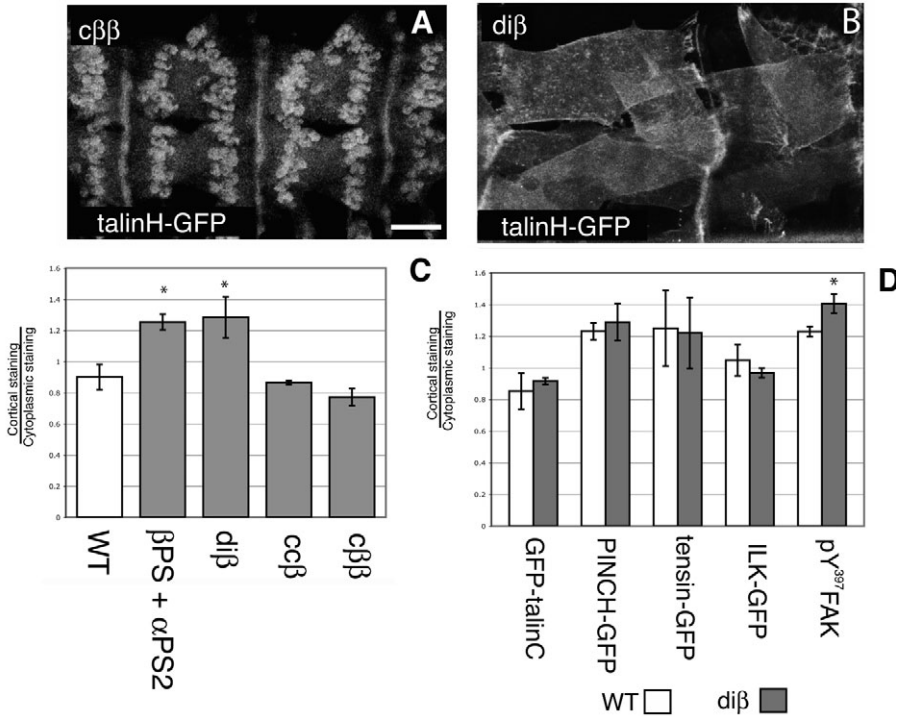

Fig. 3. Chimeras that contain oligomerized, but not monomeric, cytoplasmic domain of the integrin β subunit recruited talin head and FAK, but not other integrin-associated proteins. (A) A chimera containing the extracellular domain of monomeric CD2 fused to the cytoplasmic and transmembrane domains from βPS (cββ), did not increase talinH-GFP recruitment to the muscle ends. (B) The 'diβ' chimera, containing extracellular and transmembrane domains of the oligomeric and/or dimeric protein Torso, recruited talinH-GFP out of the nucleus to the cortex. (C) Bar graphs show the average ratio of cortical to cytoplasmic GFP staining in living embryos for wild-type muscles and muscles overexpressing integrin and integrin cytoplasmic tail chimeras. Overexpression of integrins or expression of the diβ chimera resulted in the recruitment of talinH-GFP to the cell cortex whereas expression of the cββ and ccβ chimeras did not. (D) Examination of other proteins showed that the majority were not recruited by diβ and the ratio of cortical to cytoplasmic stain was not changed between wild-type muscle and muscles that expressed diβ. However, phosphorylated FAK was weakly recruited to the cortex by diβ.

tensin, and phosphorylated focal adhesion kinase (FAK) (Clark et al., 2003; Grabbe et al., 2004; Torgler et al., 2004; Zervas et al., 2001). We calculated the ratio of cortical to cytoplasmic staining in wild-type muscles and in muscles that expressed diβ for each of these molecules and found that none of them showed increased recruitment to the cell cortex (Fig. 3D). In addition, GFP-talinC fusion protein was also not recruited to the cell cortex in greater amounts (Fig. 3D). These results showed that diβ cannot recruit to the cell cortex the full complement of integrin-associated proteins, further supporting the view that the talin head interacts directly with integrin and suggesting that tension contributes to recruitment of additional integrin-associated proteins. However, the recruitment of phosphorylated FAK to the cell cortex showed a small but significant increase of 14% by diβ (Student's *t*-test $P \leq 0.001$) (Fig. 3D). This result is consistent with recent observations in mammalian cells where FAK activation usually occurs when talin is depleted by treatment with small interfering RNA (siRNA), although paxillin and vinculin failed to be recruited to focal contacts (Giannone et al., 2003).

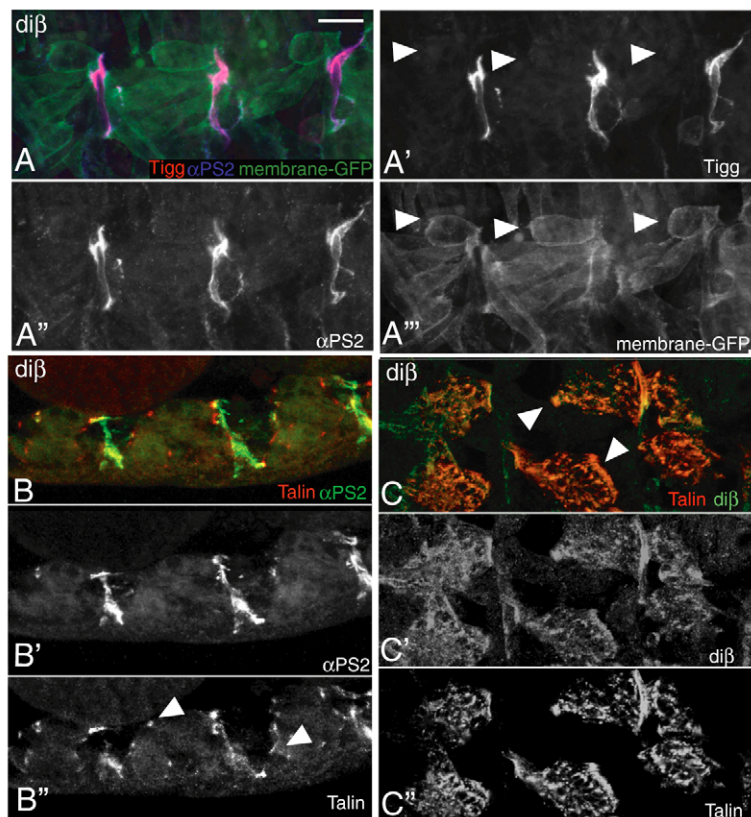Since diβ might exert its dominant negative effect by

Fig. 4. diβ interferes with the endogenous integrin-cytoskeleton link and recruits talin. (A-C'') The dominant negative phenotype in muscles expressing diβ. (A) Integrin (anti-αPS2, blue in A and white in A'') remained with the tendon matrix (anti-tiggrin, red in A and white in A') rather than at the ends of the detached muscles (myristylated-GFP, (green in A and white in A''')). By contrast, talin was found at the ends of the detached muscles (red in B, arrowheads in B''), distinct from αPS2 (green in B and B') and colocalized with diβ (C). The later-stage muscles (C) contained intracellular accumulations of talin and diβ that were not seen in earlier-stage muscles (B). Arrowhead in A', A''', C indicate ends of detached muscles. Bar, 20 μm.
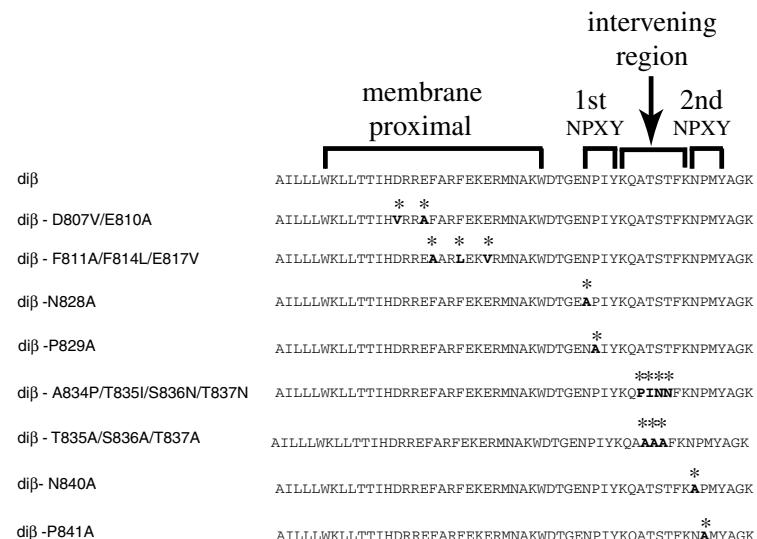

Fig. 5. Point mutations generated in the βPS cytoplasmic tail within the dimerized chimera (diβ).

competing for endogenous talin, we analyzed diβ-expressing muscles further. Fig. 4A shows embryos in which we have visualised the plasma membrane with myristylated-GFP, αPS2βPS and its ECM ligand tiggrin. It can be seen that the detached muscles did not contain integrin at their ends, which instead remained associated with the ECM (Fig. 4A''). This is similar to what was observed in embryos lacking talin (Brown et al., 2002) and in both cases suggests that integrins lost their link to the cytoskeleton. In contrast to the loss of talin in the absence of integrins (Brown et al., 2002), talin was maintained at the muscle ends (Fig. 4B''), suggesting that it is maintained by binding diβ. In fully detached muscles later in development, diβ was found both inside the cell and at the ends of detached muscles, and also colocalized with talin (Fig. 4C). In summary, talin was recruited by the diβ construct, and expression of diβ mimicked the phenotype caused by loss of talin, where the detaching muscles appeared to leave the integrins behind, which were still attached to the ECM. This supports the idea that the dominant negative effect of the diβ construct is caused by binding to talin and competing it away from the endogenous integrins. To test this further, we made a number of point mutations in the βPS cytoplasmic domain, within the diβ construct.

Residues within the βPS cytoplasmic domain required for dominant negative activity and recruitment of the talin head domain are not equivalent

The cytoplasmic tail of integrins has been subjected to extensive mutagenesis (for review see Liu et al., 2000). We made representative mutations in four regions: the membrane proximal region, the first NPxY motif, the intervening region between the two NPxYs and the second NPxY motif (Fig. 5). Only one of these mutations showed the dominant negative activity of diβ, as assayed both by muscle detachment when expressed in the muscles, and wing blisters when expressed in the wings (Table 1, data not shown). We tested multiple transgenic lines of each mutant that were expected to have some variability in the level of expression, and no differences were observed between lines. We confirmed by antibody staining that each mutant form was expressed at reasonable levels. The integrin βPS cytoplasmic tail itself contains sufficient information to be localised to muscle ends when fused to CD2 (Martin-Bermudo and Brown, 1996). This is also true of the diβ chimera and all its mutants (e.g. Fig. 6B,C). Slight variations in the level of intracellular staining of the diβ mutants was observed, indicating that some mutants might be transported to the plasma membrane less efficiently than others. However, of all mutant forms some protein from was found at the muscle ends. Moreover, western blot analysis showed that some of the diβ mutants that had lost activity were expressed at higher levels than the original diβ chimera (Fig. 6A). We then assayed the ability of these mutant diβ proteins

Table 1. diβ and CD2 constructs, their phenotypes and ability to recruit talinH-GFP

Construct	Dominant negative phenotype	Recruitment of talinH-GFP	Localization to muscle attachments
diβ	+	+	+
CCβ	–	–	+[†]
Cββ	–	–	+[†]
D807V/E810A	–	–	+
F811A/F814L/E817V	–	–	+
N828A	–	–	+
P829A	–	–	ND
T835A/S836A/T837A	–	+	+
A834P/T835I/S836N/T837N	+*	+	+
N840A	–	+	+
P841A	–	+	+

*Phenotype of PINN mutation is weaker when compared with diβ. Less muscle detachment is observed and this muscle detachment occurs at later stages. [†]Martin-Bermudo and Brown, 1996.

to recruit talinH-GFP, by measuring the ratio of cortical to cytoplasmic staining.

Mutations in the proximal NPxY

Evidence shows that the talin head binds the proximal NPxY motif in the cytoplasmic tail of the β integrin subunit (Liu et al., 2000). Consistent with this, mutation of Asp828 to Ala (N828A) inactivated recruitment of talinH-GFP to the cortex and dominant negative activity (Fig. 7B,D; Table 1). Mutation of the adjacent residue (P829A) produced similar results (data not shown; Table 1).

Mutations in the membrane proximal domain

The highly conserved membrane proximal HDR(R/K)EFA motif is important for binding to cell signalling molecules and for cell spreading (Bodeau et al., 2001; Schaller et al., 1995). A mutant diβ construct containing either of two sets of substitution mutations, D807V-E810A or F811A-F814L-E817V, within or overlapping with the HDR(R/K)EFA motif, no longer recruited talinH-GFP to the cell cortex or caused muscle detachment (Fig. 7D, Table 1). These findings show that the proximal region is also important for talinH-GFP recruitment and that, so far, the ability to recruit talinH-GFP correlates with dominant negative activity.

Mutations in the intervening region and the second NPxY

The roles of these two regions in the interaction of talin with the β cytoplasmic domain are less clear. The two regions are altered in the alternatively spliced variant of mammalian β1 cytoplasmic domain of integrin (called β1D), which has increased binding to talin; and differences in the intervening region between the two NPxY motifs account for the higher affinity of integrin β7 for filamin (Calderwood et al., 2001). At the centre of this region is the sequence ATST (at residues 834-837), which is implicated in the control of cell spreading (Bodeau et al., 2001). An ATST to PINN mutation that partly mimics the β1D isoform was generated in the *Drosophila* integrin cytoplasmic tail. However, this change did not detectably improve the ability of diβ to recruit talinH-GFP to the cell cortex (Fig. 7D) and, contrary to expectation, it reduced the dominant negative activity. Mutation of residues 835-837
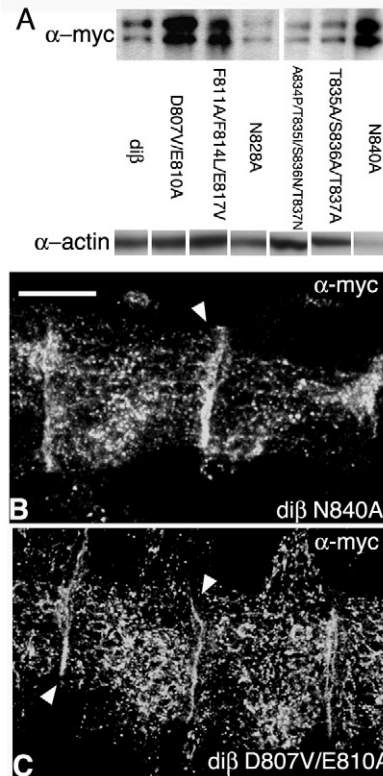

Fig. 6. Expression levels and localization of mutant forms of diβ to muscle ends. (A) Western blot analysis shows relative levels of expression of diβ and its mutated variants as expressed under the control of the *mef2::Gal4* driver; actin is used as a loading control. The diβ protein has 12 potential extracellular glycosylation sites and is similar to the wild-type Torso protein (Sprenger and Nusslein-Volhard, 1992), two bands are detected for diβ. (B,C) Antibody staining reveals that the mutant forms of diβ still localized to muscle ends (arrowheads) as illustrated for the N840A and D807V/E810A mutations. Bar, 20 μm.

from TST to AAA also did not alter recruitment of talinH-GFP to the cell cortex, but completely eliminated dominant negative activity (Fig. 7C,D, Table 1). The role of the second NPxY motif is not well defined, but mutations in it reduced focal adhesion targeting of integrins in mammalian cells without impairing talin binding (Reszka et al., 1992; Vignoud et al., 1997). Consistent with this, mutations in the second NPxY motif (either N840A or P841A) still recruited talinH-GFP, yet also eliminated dominant negative activity (Fig. 7D, Table 1).

The mutations in the C-terminal part of the βPS cytoplasmic domain have, therefore, provided unexpected results that contradict the idea that the dominant negative effect of diβ is owing to talin sequestration, because they still recruit talinH-GFP but no longer have inhibitory activity. A possible explanation is that these mutations inactivate the ability of diβ to sequester endogenous talin, even though they still bind talinH-GFP. Therefore, we examined their ability to recruit endogenous talin. This is difficult to assess, because the majority of talin is already recruited by endogenous integrins. In the presence of dominant negative activity of diβ, we found talin at the ends of the detaching muscles that lacked integrin (Fig. 4B''), but because the C-terminal mutations in diβ do not cause muscle detachment, we cannot use this assay. In the
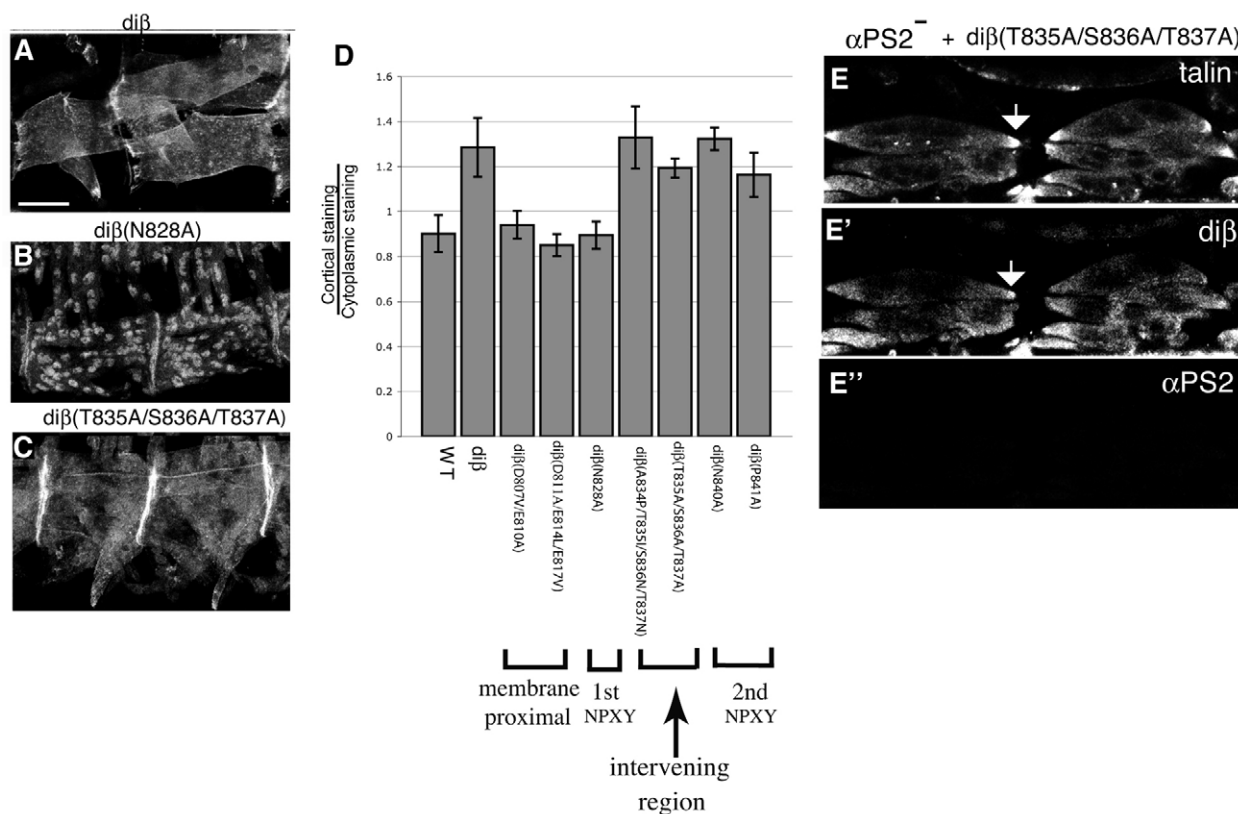
Fig. 7. Recruitment of talin head by diβ mutants. (A) diβ recruited talinH-GFP from the nucleus to the cell cortex of muscles of living late-stage embryos. (B) Mutating the proximal NPXY motif abolished this cortical recruitment in muscle. (C) A mutated ATST sequence in the integrin cytoplasmic tail still recruited talin head out of the nucleus and onto the cortex. (D) Quantification of the average ratio of cortical to cytoplasmic staining from multiple muscles showed that mutations in the ATST sequence of the integrin cytoplasmic tail or in the distal NPxY motif still recruited talin head to the muscle cell cortex, whereas mutations in the proximal region or the first NPxY did not. (E-E") In the absence of endogenous integrins (E", lacking of αPS2) the diβ mutant with a TST to AAA mutation in the integrin cytoplasmic tail (E') recruited endogenous talin to the muscle ends (E, arrow). Bar, 20 μm.

absence of the αPS2 subunit, talin fails to be recruited to the muscle ends (Brown et al., 2002), so we tested whether diβ containing the TST to AAA mutation was able to recruit endogenous talin in muscles lacking αPS2, and found that it did (Fig. 7E).

A further explanation for the loss of dominant negative activity could be that the mutant diβ proteins recruit less talin than the wild-type diβ, below a threshold of sequestration required for the phenotype. Three observations argue against this. First, we did not detect a difference in the ability of wild-type and C-terminal diβ constructs to recruit talinH-GFP out of the cytoplasm and nuclei – within the limits of quantitation provided by GFP fluorescence. Second, we can clearly score weaker dominant negative phenotypes than the one produced by wild-type diβ, such as those produced by diβY or excess βPS subunit (as described above) and, therefore, there is not a sharp threshold in dominant negative activity. Third, we tested whether reduction of talin or αPS2 levels (embryos heterozygous for a null mutation) 'sensitizes' the system, so that the C-terminal mutants now produces a dominant negative phenotype, but they still failed to produce any muscle detachment (data not shown). Thus, these experiments suggest that it is not sequestration of talin that causes the phenotype, but association with another protein. To test whether talin

sequestration has any role in the dominant negative phenotype of diβ, we overexpressed talin and found that this reduced the severity of the diβ phenotype (data not shown), suggesting talin sequestration does contribute.

Complementation between diβ mutants shows that independent interactions are required for dominant negative activity

Our experiments have suggested that recruitment of at least two proteins by diβ contributes to its dominant negative activity: talin and a second protein that binds to the C-terminus. If the interaction of these two proteins on the cytoplasmic domain is independent, we expect to see complementation between the different mutants, such that a mixture of diβ molecules carrying different mutations could recruit both proteins and cause a phenotype. Because of the large number of combinations, we first assayed the more easily scored wing-blister phenotype of diβ. The most significant complementation was found between the N828A mutation in the first NPxY motif and the P841A mutation in the second NPxY motif (Fig. 8D), with the combination of these two restoring a milder version of the wing-blister phenotype caused by wild-type diβ (Fig. 8A). However, N828A did not complement the second mutation in the second NPxY motif
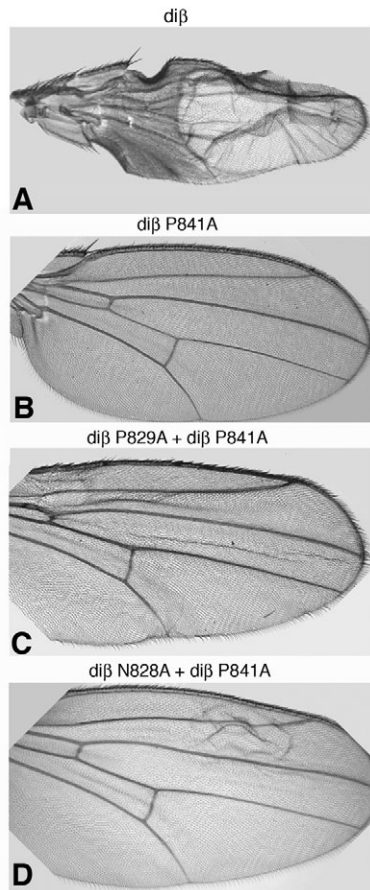
Fig. 8. Complementation between different diβ point mutations. Expression of diβ in the wing caused a dominant negative wing-blister phenotype (A) that was lost in the mutant diβ constructs (B), e.g. P841A. Dominant negative activity was restored by coexpression of two different diβ mutants. The diβ mutants P829A and P841A produced a weak wrinkled-wing phenotype (C), whereas N828A and P841A restored a wing-blister phenotype (D).

(N840A, data not shown), and the second mutation in the first NPxY motif (P829A) produced a weaker wrinkled-wing phenotype in combination with P841A (Fig. 8C). We then examined the N828A and P841A combination in the embryonic muscles, but could not detect a muscle-detachment phenotype (not shown). We suspect that this is because muscle attachment is less sensitive to disruption than adhesion in the wing, but it also possible that the dominant negative phenotype in the two tissues occurs by different mechanisms. Nonetheless, the observed complementation supports the idea that the dominant negative activity of diβ involves the recruitment of at least two cytoplasmic proteins.

Discussion

For most integrin heterodimers, the majority of interactions with cytoplasmic proteins are made by the 47-residue cytoplasmic domain of the β subunit. This short peptide has been subjected to extensive study, but we still lack a clear model of how it functions. Recent work by a number of groups has highlighted the importance of the large cytoskeletal linker talin, which was the first protein to be identified that binds to the β tail. Here, we have focused on the interaction between two domains of talin and the cytoplasmic domain of the β subunit, using the *Drosophila* embryo as our test tube. This has provided new insights into the early steps in the linkage between integrins and the cytoskeleton.

Using live imaging within the intact animal, we have provided in vivo evidence to support a direct interaction between the head domain of talin and the cytoplasmic tail of the β subunit. We found that this interaction requires an 'alteration' to the cytoplasmic domain of integrin because only one of three chimeric transmembrane proteins containing this domain was able to recruit the talin head. Since the active chimera is derived from a constitutively active receptor tyrosine kinase, it seems likely that its ability to constitutively dimerize or oligomerize accounts for its special activities, leading us to refer to it as diβ. Previous work showed that diβ was constitutively active in sending integrin signals that regulate gene expression (Martin-Bermudo and Brown, 1999), and we have shown here that it also acts as a dominant negative protein on integrin-mediated adhesion at muscle attachment sites. We found that the dominant negative phenotype of diβ consists of a detachment between integrins and the cytoskeleton, similar to the phenotype seen in the absence of talin (Brown et al., 2002). This suggests an explanation for the dominant negative activity of diβ: it sequesters talin away from the endogenous integrins. Consistent with this, diβ recruited talin and TalinH-GFP, but not other proteins required for integrin adhesion, such as PINCH, ILK and tensin. In addition, this latter finding further supports the direct interaction between integrins and talin, and shows that recruitment of talin is not sufficient to trigger the assembly of the whole complex of proteins that contribute to the link between integrins and the cytoskeleton.

Based on these observations, it was possible to propose a simple model where talin is the only protein recruited directly by integrins and the dominant negative activity of diβ is solely due to talin sequestration. We tested this by generating point mutations in the β subunit of the cytoplasmic domain within the diβ chimera and by assaying their ability to cause muscle detachment and recruit TalinH-GFP, surmising that, if this model was correct there should be a clear correlation between the two. However, this proved not to be the case. Mutations throughout the length of the integrin tail caused a loss of dominant negative activity, but only those in the half closest to the membrane could not recruit TalinH-GFP. This leads us to hypothesize that a factor X binds to the second half of the integrin cytoplasmic domain and contributes to the dominant negative effect.

Direct versus indirect interaction of different talin domains with integrins in vivo

Based on previous in vitro studies it was proposed that the head domain of talin acts as the preferred site for integrin binding (Calderwood et al., 1999; Yan et al., 2001). The talin head domain, which in vertebrates can be found endogenously due to cleavage by the calcium dependent protease calpain (Beckerle et al., 1987), has also been shown to mediate integrin activation (Calderwood et al., 1999; Yan et al., 2001). In this study, we showed that the talin head can localize to the muscle attachments in an integrin-dependent manner and that expression of dimeric integrin cytoplasmic tail chimeras is

sufficient to recruit it to the cell cortex. Experiments in myoblasts and the direct correlation between levels of integrin and talinH-GFP recruitment strongly suggest that, in vivo recruitment of talinH-GFP by the integrin cytoplasmic tail occurs independently of any accessory factors and, therefore, is most probably direct. The finding that talinH-GFP was the only protein tested that was found around the entire cortex of muscles containing diβ or excess integrin also supports a direct interaction between them. There might be a difference between the membrane domains on the lateral sides of the muscles versus the ends, which blocks recruitment of additional integrin associated proteins to the lateral sides.

Further support for a direct interaction between the βPS cytoplasmic domain and the talin head domain in vivo came from our mutational analysis. Those residues essential to recruit talinH-GFP in vivo are within the same regions where the cytoplasmic domain binds to talin in vitro. Thus, the first NPxY motif of the β integrin cytoplasmic tail is crucial for binding to the talin head domain both in vivo and in vitro (Pfaff et al., 1998; Garcia-Alvarez et al., 2003). Both approaches show that talin binding requires more than just this region. For example, peptides that correspond to a small region covering the NPLY motif of β3 integrin and the preceding eight amino acid residues were unable to bind to talin by themselves in comparison to peptides covering the membrane proximal region of the tail (Patil et al., 1999). This led to the suggestion that the NPLY domain by itself can mediate talin binding in the context of the full-length cytoplasmic tail but that the membrane proximal region is sufficient by itself. NMR studies showed that, the region of the integrin cytoplasmic tail that is perturbed upon binding of the talin head includes residues in the membrane proximal region and this is abolished when NPxY is mutated (Ulmer et al., 2003). Peptides only containing the region that includes the NPxY region showed a surprisingly low binding affinity for the talin head, about 100 times less than the affinity for PIP4,5-kinase (Barsukov et al., 2003). Our in vivo results support the idea that talin binding requires interaction with multiple regions of the integrin cytoplasmic tail either simultaneously or sequentially.

In contrast to the talin head, the C-terminal domain of talin behaved quite differently. It localized to sites of adhesion and was also specifically and strongly recruited to the developing Z-lines. Like the talinH-GFP, the C-terminal region required integrins for its localization but, by contrast, it was not recruited by overexpression of integrins or diβ, nor was it recruited more efficiently when the levels of endogenous talin were reduced, instead it required talin for its localisation. This is not too surprising because GFP-talinC does not include the recently defined C-terminal integrin-binding domain (Tremuth et al., 2004).

Since GFP-talinC contains the I/LWEQ domain, which is thought to have actin-binding activity, its failure to colocalise with actin filaments is surprising. In contrast to other fusion proteins of GFP to actin-binding domains, we have tested, the talin C-term does not decorate actin filaments in the developing muscle (N.H.B., unpublished observations). Instead, it decorates the Z-lines and muscle ends, with a distribution that is very similar to that of the N-terminal domain of tensin, which also binds actin in vitro (Torgler et al., 2004). Recently it was reported that the actin-binding site is cryptic, supporting

our findings (Senetar et al., 2004). Some possible candidates for the recruitment of this domain seem unlikely, such as vinculin and talin itself. GFP-talinC does not include any of the identified vinculin-binding sites (Hemmings et al., 1996). The talinC domain does contain a recently identified homodimerization site (Senetar et al., 2004), but if it was recruited solely by dimerizing with endogenous talin, we would not expect such strong Z-line relative to muscle-end staining because talin is much more enriched at the muscle ends. In addition, we would expect it to be recruited by talin associated with diβ, which it is not. Therefore, the identity of the molecules that mediate the localization of GFP-talinC remains a topic for further study.

Does the cytoplasmic tail of the integrin β subunit need to oligomerize to recruit talin?

We found that different chimeric proteins containing the βPS cytoplasmic domain did not have an equal ability to recruit talinH-GFP to the cell membrane and, thus, the extracellular domain of the chimera influences intracellular interactions. One clear difference is that, using extracellular domains from a receptor tyrosine kinase that has been mutated so that it signals independently of ligand-binding resulted in recruitment of talinH-GFP. The strength of the constitutive signalling produced by these mutant receptors correlated with their ability to recruit talinH-GFP as chimeric proteins. These findings point to dimerisation or oligomerisation as a key trigger for talin recruitment. This fits with the recent discovery of homotypic interactions between the transmembrane domains of integrin subunits, leading to a model where ligand-bound integrin heterodimers exist in a complex-cluster on the cell surface. There, the transmembrane and cytoplasmic domains of the β subunit form trimers and those of the α subunits form dimers in the membrane, whereas outside the cell αβ heterodimers bind to ligand (Li et al., 2003). The increased recruitment of talinH-GFP to the dimers or trimers of the β cytoplasmic domain could occur by cooperative binding, which would require some interaction between different talin head domains, or the oligomers could stabilise a conformation of the β cytoplasmic domain that binds tightly to the talin head. This latter model gives an alternate explanation: the chimeras with the mutant forms of the receptor tyrosine kinase could induce the βPS cytoplasmic domain to adopt a different conformation compared with the CD2 chimera, which bind to talin more tightly. At present, we lack the tools to unambiguously distinguish between these different possibilities, but we favour oligomerization as our working model.

The formation of β subunit oligomers might also explain why overexpression of the βPS integrin subunit was dominant negative on integrin adhesion – similar to that produced by diβ. Formation of oligomers can be suppressed by co-overexpressing the αPS2 subunit, indicating that it is not overexpression of integrin heterodimers that is the problem, but free β subunits. One explanation is that free βPS subunits form – just like diβ – homodimers or homotrimers at low levels, which can be transported to the plasma membrane where they recruit cytoplasmic proteins but cannot mediate extracellular adhesion. This might also explain why the dependence on dimerization for dominant negative activity that we observed has not been reported in other systems, because even a low level of dimerization may induce dominant negative activity.

Dominant negative activity of the integrin cytoplasmic domain in chimeric transmembrane proteins

In the introduction, we proposed two models that explain the dominant negative effect of chimeric proteins containing the integrin β subunit cytoplasmic tail: a feedback model, involving excess signalling, and a competition model, involving sequestration of key cytoplasmic proteins required for endogenous integrin function. Our results suggest that sequestration of talin accounts for some but not all of the dominant negative effect. Talin is one of the few integrin-associated proteins tested that is recruited by diβ and its overexpression partially suppressed the dominant negative activity. Mutants of diβ that lost talinH-GFP binding also lost dominant negative activity. However, mutations in the C-terminal part of the β tail still recruited talin yet also lost dominant activity, suggesting involvement of another protein. We therefore hypothesize that, binding of a factor X to the C-terminus is also required for the dominant negative effect. The requirement for two factors received some support from our finding that coexpression of certain pairs of diβ mutants partially restored dominant negative activity; our thinking is that the heterodimers formed can now recruit both talin and factor X.

We find it hard to explain how diβ could only produce a dominant negative effect when sequestering two proteins, because we would expect that recruiting a single protein has some effect, and recruiting both has an additive effect. Instead we see very strong synergy, which is easier to explain if factor X has a signalling role. For example, one speculative model is that factor X is a kinase that phosphorylates and inactivates talin when it is bound at the adjacent site on the β cytoplasmic tail. Exchange of the inactivated talin would then lead to a gradual inactivation of the cytoplasmic pool of talin, which could be partially alleviated by increasing the amount of talin by overexpression. In the case of the endogenous integrins, there would have to be a mechanism to inactivate this inhibition. This could be achieved by one of the integrin-associated proteins that is recruited by the endogenous integrins but not by the diβ-inactivating factor X or by removing the inhibitory phosphate from talin. The inactivation of the inhibition by endogenous integrins must not be efficient enough to counter the negative effect of diβ. Thus, in the end our best model combines both sequestration and excessive signalling.

A number of candidates for factor X that interact with the relevant region of the β subunit cytoplasmic domain have already been identified, including filamin (Calderwood et al., 2001), PKCα (Parsons et al., 2002), non-muscle myosin (Jenkins et al., 1998), and Src (Arias-Salgado et al., 2003). Non-muscle myosin can be recruited by the CD2βPS chimera (Bloor and Kiehart, 2001). Src is activated by binding to the C-terminal region the of the β3 integrin cytoplasmic tail, and this is enhanced by clustering and homo-oligomerization of the integrins (Arias-Salgado et al., 2003). The role of Src in integrin function in *Drosophila* has yet to be elucidated. Phosphorylated FAK was recruited by diβ, and overexpression of FAK causes muscle detachment (Grabbe et al., 2004). By contrast, sequestration of FAK is unlikely to cause a defect because the absence of FAK did not cause defects in integrin-mediated adhesion in *Drosophila* (Grabbe et al., 2004). Thus, there are candidate kinases that interact with this region of the β tail and could send inhibitory signals.

Recruitment ability of the integrin cytoplasmic domain in chimeric transmembrane proteins

We have found that when the integrin β tail is placed on a heterologous transmembrane protein it only recruited some integrin associated proteins. In particular, talin and FAK were recruited, but not ILK, PINCH or tensin. This suggests additional input, required to assemble the full complement of proteins that contribute to the integrin-cytoskeleton link. Either of the two domains missing from these chimeras could provide the input: the extracellular ligand-binding domain, composed of both α and β subunits, or the α subunit cytoplasmic domain. Since an integrin heterodimer lacking the α subunit cytoplasmic domain can mediate muscle attachment (Martin-Bermudo et al., 1997), we favour the extracellular ligand-binding domain. This could either provide unique conformational changes to the β tail, or it could allow tension to be placed on the integrin-cytoskeletal linkage.

Materials and Methods
Drosophila strains
The following null alleles were used if^{B4}, mys^{XG43}, $rhea^{79A}$ (Brown et al., 2002). Gal4 drivers used were *24B::Gal4* or *mef2::Gal4* for muscle and *69B*::Gal4 for wing. The UAS lines used were *UAS::diβ* (Martin-Bermudo and Brown, 1999), *UAS::βPS*, *UAS::CCβ* and *UAS::Cββ* (Martin-Bermudo and Brown, 1996), *UAS::αPS2* (Martin-Bermudo et al., 1997), ILK distribution was examined with a GFP-tagged gene (Zervas et al., 2001).

Molecular biology
Mutant diβ constructs were generated starting with the original UAS-diβ (Martin-Bermudo and Brown, 1999). Site-directed mutagenesis to introduce modification into the integrin cytoplasmic tail was performed with standard methods. For the talinH-GFP construct, the talin N-terminal end was PCR-amplified from genomic DNA with the primer talin-H FWD (5′-GATTGGTACCGATGGTTCCGGGTTT-CGG-3′) that starts 457 bases upstream of the talin ATG and introduces a *Kpn*I site. The complimentary primer talin-H BWD (5′-GTGGAATTCCTCCACATTTAGTT-GCTCCAT-3′) starts 36 bases downstream of the putative Calpain cleavage site at the boundary between talin head and rod and introduces an *Eco*RI site. The fragment was cloned into pUASp-PL mGFP C-terminal fusion vector (gift of Uwe Irion The Wellcome Trust/Cancer Research UK Gurdon Institute, Cambridge, UK) using the *Kpn*I and *Eco*RI sites.

For the GFP-talinC construct the talin C-terminal end was PCR-amplified from genomic DNA with the primer talin-Tail FWD (5′-GATACTGCAGACAGTCATT-GCTGAGAACGAG-3′) starting 478 base pairs before the start of the ILWE/Q domain containing a *Pst*I site, talin-Tail BWD (5′-AGTGCCGCGGAGTATGTTT-ATGGGTGTGTA-3′) starts 102 base pairs downstream of the talin termination codon containing a *Ksp*I site. The fragment was cloned into pUASp-PL mGFP N-terminal fusion vector (gift of Uwe Irion) using the *Pst*I and *Ksp*I sites.

Production of anti-talin head antibody
A fragment containing amino acids 317to 434 was cloned into pGEX-2T GST fusion vector (Pharmacia). The soluble protein was purified on glutathione agarose and eluted. The protein was used to immunize rabbits (Eurogentec) and the serum affinity purified.

Fixation and antibody staining
Antibody staining was carried out according to standard procedures. The following antibodies were used: anti-βPS (CF6G11 mouse monoclonal antibody (mAb), 1:10, Developmental Studies Hybridoma Bank), anti-αPS2 (7A10 rat mAb, 1:10), α-talin (E168, mouse mAb, 1:10) (Brown et al., 2002), anti-GFP (rabbit polyclonal, 1:500, Promega), anti-CD2 (OX-34 mAb, 1:500, Serotec), anti-Tiggrin (mouse polyclonal, 1:500) (Fogerty et al., 1994), anti-FAK[pY^{397}] (rabbit polyclonal, 1:500, Biosource). The diβ constructs are tagged with a myc epitope at the N-terminus (following the signal peptide) and were detected with anti-Myc (mAb, 1:1000, Santa Cruz Biotechnology). Confocal images were collected using a Biorad Radiance 2000/NikonE800 microscope with 40×/1.30 oil and 60×/1.40 oil objectives. Images were processed using Adobe Photoshop.

Western blot analysis
Protein was isolated from embryos and blotted according to standard methods.

Antibodies used were anti-Myc (mAb, 1:5000, Santa Cruz Biotechnology), anti-GFP (rabbit polyclonal, 1:5000, Promega), anti-actin (C4 mAb, 1:500, Abcam).

Data collection from images

All image analysis was done using the imageJ software. Representative samples of at least five muscles were analyzed for each genotype. The average staining intensity was measured for the entire cell cortex as well as for the entire cytoplasm of the cell for each muscle; and a ratio was calculated and an average ratio and standard deviation was determined. The statistical analysis was carried out in Excel.

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