Analysis of PINCH function in Drosophila demonstrates its requirement in integrin-dependent cellular processes

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SUMMARY

Integrins play a crucial role in cell motility, cell proliferation and cell survival. The evolutionarily conserved LIM protein PINCH is postulated to act as part of an integrin-dependent signaling complex. In order to evaluate the role of PINCH in integrin-mediated cellular events, we have tested directly the in vivo function of PINCH in Drosophila melanogaster. We demonstrate that the steamer duck (stck) alleles that were first identified in a screen for potential integrin effectors represent mutations in Drosophila pinch. stck mutants die during embryogenesis, revealing a key role for PINCH in development. Muscle cells within embryos that have compromised PINCH function display disturbed actin organization and cell-substratum adhesion. Mutation of stck also causes failure of integrin-dependent epithelial cell adhesion in the wing. Consistent with the idea that PINCH could contribute to integrin function, PINCH protein colocalizes with βPS integrin at sites of actin filament anchorage in both muscle and wing epithelial cells. Furthermore, we show that integrins are required for proper localization of PINCH at the myotendinous junction. The integrin-linked kinase, ILK, is also essential for integrin function. We demonstrate that Drosophila PINCH and ILK are complexed in vivo and are coincident at the integrin-rich muscle-attachment sites in embryonic muscle. Interestingly, ILK localizes appropriately in stck mutant embryos, therefore the phenotypes exhibited by the stck mutants are not attributable to mislocalization of ILK. Our results provide direct genetic evidence that PINCH is essential for Drosophila development and is required for integrin-dependent cell adhesion.

Key words: LIM domains, Cell adhesion, Cytoskeleton, Integrins, Drosophila

INTRODUCTION

Cell adhesion to the extracellular matrix (ECM) is required for tissue architecture and can have dramatic effects on cell behavior (DeSimone, 1994; Gotwals et al., 1994; Dedhar and Hannigan, 1996; Giancotti, 1997). Integrins are transmembrane, heterodimeric receptors that comprise the primary recognition sites for binding to ECM (Hynes, 1992; Hynes, 2002). α- and β-integrin subunits possess large extracellular domains that form a binding interface for specific ECM components (Adams and Watt, 1993; Humphries and Newham, 1998). The cytoplasmic domains of integrins tether actin filaments, and recruit a wide array of proteins involved in signal transduction (Critchley et al., 1999; Calderwood et al., 2000; Giancotti, 2000; Yamada and Even-Ram, 2002). Proteins that associate either directly or indirectly with integrin cytoplasmic tails may also contribute to activation of the ligand binding capacity of the integrins, thus modulating integrin adhesive function by an ‘inside-out’ signaling mechanism (Ginsberg et al., 1992; Hughes and Pfaff, 1998; Liddington and Ginsberg, 2002).

One cytoplasmic protein that has been postulated to play a role in integrin function is PINCH, a protein comprising five tandemly arrayed LIM domains (Rearden, 1994). LIM domains are double zinc-finger structures that serve as protein-binding interfaces (Michelsen et al., 1993; Schmeichel and Beckerle, 1994); therefore, PINCH probably functions as a molecular scaffold that supports the assembly of a multi-protein complex at sites of integrin enrichment. In agreement with this notion, biochemical studies of human PINCH have identified Integrin-Linked Kinase (ILK) as a binding partner for the first LIM domain of PINCH (Tu et al., 1999), and the SH2-SH3 adaptor protein NCK2 as a partner for the fourth LIM domain (Tu et al., 1998). Although the complete binding partner repertoire of PINCH remains to be elucidated, the colocalization of PINCH with integrins and its capacity to bind ILK and NCK2 provided the first hints that PINCH might contribute to integrin function (Wu, 1999; Wu and Dedhar, 2001).

Further support for the view that PINCH is essential for integrin function came from studies in which PINCH expression in C. elegans was compromised by RNA interference. Developing embryos that are deficient in PINCH display a paralyzed-at-twowold (PAT) phenotype, similar to that observed in integrin mutants (Hobert et al., 1999). In spite of the comparable developmental arrest when either integrin or PINCH function is compromised in the worm, this phenotypic
description did not provide mechanistic insight into the relationship between PINCH and integrins. Recently, however, it was demonstrated that expression of a dominant-negative form of PINCH in tissue culture cells results in compromised cell adhesion (Zhang et al., 2002c). These findings are consistent with the view that PINCH is required for integrin-dependent cell adhesion. However, because the LIM domain is a conserved structural feature found in many modular proteins (Schmeichel and Beckerle, 1994; Dawid et al., 1998; Bach, 2000), it is essential that conclusions from studies using dominant-negative tools be confirmed using a loss-of-function strategy where specificity is insured.

We have taken a genetic approach in Drosophila to define the physiological contributions of PINCH to integrin-mediated cellular events in vivo. Drosophila provides an excellent model system with which to study integrin function as integrin-dependent cell adhesion is required for proper organization of multiple embryonic and adult tissues (MacKrell et al., 1988; Brower and Jaffe, 1989; Leptin et al., 1989; Brabant and Brower, 1993; Brown, 1994; Brower et al., 1995). Moreover, Drosophila is a particularly valuable system for assessing PINCH function because, in contrast with C. elegans and mouse, where multiple PINCH family members are present (Hobert et al., 1999; Zhang et al., 2002a), only a single pinch gene exists in the fly. Our analysis of the cellular and developmental consequences of mutations in Drosophila pinch illustrates that PINCH is essential for integrin-dependent cell adhesion events in embryos and adults and reveals that PINCH is required to stabilize membrane-cytoskeletal linkages at sites of cell-substratum anchorage.

MATERIALS AND METHODS

Drosophila stocks and genetics

All stocks were reared under standard laboratory conditions at 25°C unless otherwise noted. The stck parental strain 82w+ or w1118 served as wild-type controls in all experiments. The f(3)097 stock contains a P-element insertion at 85A1, ~50 kb distal to stck, and an associated deletion of DNA that removes stck plus at least two other vital genes. mvyX043 and iP2 are null mutations in βPS integrin and αPS2 integrin, respectively (Bunch et al., 1992; Brown, 1994). Embryos that lack both maternal and zygotic wild-type respectively (Bunch et al., 1992; Brown, 1994). Embryos that lack unless otherwise noted. The balancer chromosome, was sequenced directly with Drosophila published elsewhere (Prout et al., 1997). Injection of transgenes into mitotic recombination (Xu and Rubin, 1993), following procedures of amplification products from both the . The PCR reaction, representing a mixed population standard PCR reaction using primers that would amplify the coding stck Genomic DNA from all cloning and other DNA manipulation was carried out essentially (Spradling, 1986).

Molecular biology

All cloning and other DNA manipulation was carried out essentially as described (Sambrook, 1989), with exceptions noted below. Genomic DNA from stck heterozygous flies was subjected to a standard PCR reaction using primers that would amplify the coding region for pinch. The PCR reaction, representing a mixed population of amplification products from both the stck chromosome and the balancer chromosome, was sequenced directly with pinch primers. The amplification was carried out twice, and each time the PCR reaction product was sequenced on both strands to ensure that the lesions detected were not due to a polymerase error propagated during the PCR amplification. A lesion in pinch was identified when the trace of the sequencing reaction went out of phase, indicating a point where the two PCR products differed in their sequence. This phase-shift was seen in sequencing reactions on both strands. As a control, genomic DNA from 82w+ flies was subjected to the same treatment in parallel and sequenced.

To generate the pinch genomic rescue construct, genomic DNA encompassing pinch was amplified by PCR, cloned into the TA vector (Invitrogen) and excised with SpeI and NotI. This fragment, which contains the pinch transcription unit plus 2882 bp of DNA 5’ to the transcription unit and 436 bp 3’, was cloned into the pCaSpeR4 vector (kindly provided by Carl Thummel) to generate the P[w+ pCas-pingen] transformation construct.

Northern blot and RT-PCR

Total RNA was isolated from Drosophila at various stages of development, using Trizol (Gibco BRL), following the manufacturer’s recommendations. Approximately 15 μg RNA from each sample was loaded and run on a denaturing formaldehyde gel and transferred to Gene Screen nylon membrane (PerkinElmer Life Sciences). The resulting blot was hybridized in Ultragyb (Ambion) and processed according to the manufacturer’s instructions. Band intensities were assessed by scanning the autorads and quantifying pixel values on a Kodak 440 image station (Kodak); pinch values were normalized relative to the rp49 signals to control for any unequal loading.

RT-PCR analysis of the pinch transcription unit was conducted on mRNA samples from 16- to 24-hour-old embryos, third instar larvae and adult females, using primers that would amplify the entire PINCH transcript. In each case, 50 ng of mRNA was used in a reaction according to the manufacturer’s instructions (Access RT-PCR introductory kit, Promega). Resulting cDNAs were sequenced with pinch-specific primers.

Antibody production, affinity purification, immunoprecipitation and immunodetection of proteins

Rabbit polyclonal antisera were generated (Capralogics, Hardwick, MA) against an 18 amino acid peptide (ELRRRLRTAHEMTMKKNT) corresponding to residues 318-335 of the predicted PINCH protein. Anti-PINCH antibodies were affinity-purified prior to use. PINCH complexes were immunoprecipitated with the affinity-purified anti-PINCH antibody from Drosophila 0- to 18-hour-old embryo extracts, prepared from a transgenic line carrying an ILK::GFP genomic construct (Zervas et al., 2001). Approximately 1 μg of affinity-purified anti-PINCH antibody, pre-immune serum or anti-MLP60A antisera (Stronach et al., 1996) was used for each immunoprecipitation. Recovered proteins were resolved by SDS-PAGE and analyzed by western immunoblots probed with affinity-purified PINCH polyclonal antibody at a dilution of 1:10,000, anti-GFP mAb (Clontech, Palo Alto, CA) at a dilution of 1:500, or anti-MLP60A antisera (Stronach et al., 1996) at a dilution of 1:600.

Embryos were collected and prepared for immunofluorescence analysis essentially as described (Patel, 1993). In some cases (e.g. Fig. 3), embryos were prepared for immunofluorescence by heat fixation (Miller et al., 1989), as opposed to the normal formaldehyde fixation. Developing wings were dissected from staged pupae (~45 hours after pupariation), and prepared for immunofluorescence using published procedures (Wolff, 2000). Antibodies to proteins visualized in this study were used at the following concentrations: rabbit anti-PINCH (this study) 1:500; mouse anti-β-galactosidase (Promega) 1:2000; rabbit anti-β-galactosidase (Cappel) 1:5000; rabbit anti-Mlp84B (B50) (Stronach et al., 1996) 1:500; mouse anti-βPS integrin (C6G9I11 ascites) (Brower et al., 1984) 1:1000; and rabbit anti-dPak (Harden et al., 1996) 1:500. Secondary antibodies were preabsorbed against w1118 embryos before use. For phalloidin staining, the fixation procedure was changed such that embryos were devitellinized in 80% ethanol instead of methanol. Images were obtained from a LSM-510 confocal microscope (Zeiss).
RESULTS

Drosophila PINCH is an essential gene encoded by the steamer duck (stck) locus

Drosophila PINCH displays five tandemly arrayed LIM domains that exhibit a high degree of sequence similarity to human PINCH1 (Fig. 1A). Molecular and genomic analyses confirm that there is a single PINCH gene in Drosophila. Northern blots probed with a Drosophila pinch cDNA reveal a single transcript of 1.4 kb (Fig. 1B). Genefinder programs do predict a possible alternative start site that would use a different first exon; however, this would not affect the coding sequence and no existing Drosophila PINCH ESTs contain this alternative exon (FlyBase, 1999; Rubin et al., 2000). Moreover, RT-PCR analysis of RNA from staged samples results in products identical in sequence to the original cDNA (data not shown), further supporting the view that there is only one RNA species transcribed from the pinch locus. Northern analysis of developmentally staged RNA samples (Fig. 1B) revealed that pinch expression parallels that of βPS integrin (Zusman et al., 1990). Specifically, pinch transcripts are maternally inherited and are expressed zygotically at the time of muscle differentiation. pinch RNA levels decrease during the larval stages, but increase again during pupal development, coincident with the terminal differentiation of the adult structures.

Alignment of the Drosophila pinch cDNA sequence with the deposited Drosophila genome sequence indicates that the pinch locus maps to 84E11-85A1. This assignment is in agreement with chromosome in situ hybridization data that placed pinch at 85A1-3 (data not shown). Several pre-existing mutations, which are generated from unrelated mutagenesis screens, map to the same cytological interval as pinch (FlyBase, 1999). One lethal complementation group, stck, is represented by two alleles (stck17 and stck18) that were isolated in a mutagenesis screen designed to identify gene products required for integrin function (Prout et al., 1997). Moreover, stck mutations were reported to enhance a phenotype associated with compromised integrin function (Prout et al., 1997). By DNA sequence analysis, we found that both stck alleles contain mutations in the pinch locus that were predicted to disrupt the protein-coding region (Fig. 1C). stck17 contains

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Fig. 1. Drosophila pinch is encoded by the steamer duck (stck) locus. (A) Protein sequence similarity between the LIM domains of human PINCH1 (LIMS1 – Human Gene Nomenclature Database) and Drosophila PINCH. (B) Northern blot analysis of staged RNA samples from different developmental timepoints. Embryonic stages are numbered and represent time collected after egg laying at 25°C. Larval samples (L) are from the three larval stages (instar); the third instar larval sample is represented twice to confirm the decrease in rp49 probe seen at that time of development (Borie et al., 1999). RNA markers (not shown) indicated the size of the hybridizing band to be 1.4 kb. Northern blot quantitation is indicated below by the graph. (C) Sequence analysis of the stck alleles. The pinch transcription unit contains six exons, indicated by the blocks, with the initiating MET codon encoded by the second exon. Individual LIM domains are color coded. The pinch sequence in stck17 contains a 571 bp deletion encompassing nucleotides 2095-2664 (corresponding to 615-1066 of the published cDNA sequence; Accession Number AF078907). stck18 harbors a 2 bp deletion removing nucleotides 2309-2310 in the fifth exon (774-775 of the published cDNA sequence), resulting in a frame shift in the middle of the fourth LIM domain. (D) Western blot demonstrating reduction of PINCH protein in stck mutants. Each lane contains 10 μg of protein lysate from the following samples: stage 17 wild-type embryo (lane 1); stage 17 l(3)097 homozygous embryo (lane 2); stage 17 l(3)097/stck17 embryo (lane 3); stage 17 l(3)097/stck17 embryo from stck17 germline clone (lane 4). The blot has been hybridized with the affinity-purified PINCH antiserum. The faint band present in lane 4 that migrates at ~40 kDa is nonspecific immunoreactivity.
a 571 bp internal deletion that removes DNA encoding the last two and a half LIM domains of PINCH, while stck18 has a two bp deletion that alters the reading frame in the fourth LIM domain.

The lethality associated with homozygous stck mutations can be rescued by introduction of a transgene that encodes wild-type PINCH (data not shown). Further confirmation that PINCH is encoded by the stck locus comes from western immunoblot analysis of PINCH protein levels in stck mutants (Fig. 1D). Affinity-purified antiserum directed against a C-terminal PINCH epitope recognizes a single polypeptide with an apparent molecular mass of 31 kDa in wild-type embryos (Fig. 1D, lane 1). Wild-type PINCH protein levels are significantly reduced in stck zygotic mutants (Fig. 1D, lanes 2 and 3) and the protein is undetectable when maternal PINCH is also eliminated (Fig. 1D, lane 4). Collectively, these data provide compelling evidence that Drosophila pinch is encoded by the stck locus.

Mutations in PINCH destabilize membrane cytoskeletal linkages in embryonic muscle and compromise cell anchorage

We have characterized the phenotypes associated with the two stck alleles described above. When examined as hemizygous mutations, greater than 85% of the stck mutant embryos die, indicating a strong requirement for PINCH during embryonic development. Comparison of wild-type larvae and the few stck mutant larvae that survive to hatch revealed dramatic morphological differences. The stck mutant larvae are significantly shorter than wild-type larvae (0.50±0.05 mm versus 0.72±0.03 mm). Additionally, stck mutant larvae are nearly immobile, a phenotype that suggests impaired muscle function, and die within 24 hours of hatching.

pinch transcript is expressed prominently in the developing somatic muscles of Drosophila embryos (Hobert et al., 1999), therefore we examined the mutant embryos more closely for any perturbations in somatic muscle patterning and development. Initial muscle patterning is not affected in stck mutants (data not shown), indicating that PINCH is not required for muscle cell differentiation, fusion or migration. Defects in muscle morphology are first detected in stck mutants at embryonic stage 16. By comparing wild-type and mutant embryos that are stained with antibody directed against Mlp84B, a muscle-specific protein that is associated with the contractile apparatus and enriched at muscle-attachment sites (Stronach et al., 1996), it is evident that the mutant muscles exhibit a distorted morphology (Fig. 2A,B). The embryonic musculature is less organized in stck mutants compared with their wild-type counterparts, and gaps are evident occasionally between adjacent muscle cells, indicating a failure of some muscle-attachment sites (Fig. 2B, arrowheads).

To evaluate whether the misshaped muscles had underlying cytoskeletal defects, we examined the actin organization in stck mutant embryos. In early stage 17 embryos, the actin filaments in the wild-type muscle cells are clearly organized into linear arrays that extend to the lateral borders of each muscle fiber (Fig. 2C). There is a clear

Fig. 2. Loss of PINCH function disrupts muscle morphology and actin filament organization. (A,B) Lateral views of stage 16 embryos stained with an antibody against the Mlp84B protein to visualize the somatic muscles. The stck mutant (B) displays a disruption in muscle fiber morphology. Arrowheads in B indicate areas where the muscles have lost their attachment to the tendon matrix. Arrows in A,B indicate enrichment of Mlp84B at muscle-attachment sites. (C-G) Confocal micrographs of embryonic muscle from wild-type (C,E), stck18(l(3)097) (D), stck17(l(3)097) (F) and l(3)097 homozygote (G) embryos, labeled with fluorescent-phalloidin to visualize F-actin. (C,D) Muscle fibers from early stage 17 embryos. A set of lateral muscles from two segments is shown in each panel. Actin bundles are readily distinguished in the wild-type muscles because of the precise orientation of the actin filaments in each muscle. This arrangement is not maintained in the mutant muscles (D). (E-G) Late stage 17 embryos. Note that the defects exhibited by a stck17(l(3)097) embryo (F) are similar to those from the l(3)097 homozygote (G), when compared with a wild-type embryo (E). Equivalent regions are indicated by an asterisk in the mutant and wild type.
enrichment of filamentous actin at the muscle termini, where the muscle cell membranes are attached to the tendon cell matrix. By contrast, the stick mutant muscles do not display such a high degree of actin filament organization (Fig. 2D). The actin filament bundles that comprise the myofibrils are buckled in appearance, and often do not extend to the segment boundaries. Additionally, many of the muscle attachments lack the enrichment in filamentous actin seen in wild-type animals. The significant alteration of myotendinous junction structure and composition suggested that the function of this specialized adhesive junction was probably compromised in the stick mutant embryos. The disturbed cytoskeletal organization observed in the stick mutants progressively worsens as development proceeds, such that in late stage 17 mutant embryos, actin filament arrays are largely retracted to one end of the muscle (asterisk in Fig. 2F), indicating a failure of at least one of the actin-membrane anchorage sites that normally tether the ends of the contractile machinery to the muscle cell membrane.

Both stick17 and stick18 alleles retain some PINCH-coding sequence. In particular, these mutant alleles could theoretically support the production of C-terminally truncated PINCH products that might retain partial function or have dominant-negative activity. In order to assess whether stick17 and stick18 behave as simple loss-of-function alleles, we compared the cellular phenotypes of stick17 and stick18 hemizygotes with embryos that carry a homozygous deletion of the stick locus (l(3)097) and observed a comparable terminal phenotype (Fig. 2F,G). These findings illustrate that the stick17 and stick18 alleles disrupt PINCH function to a similar extent as occurs when PINCH function is completely eliminated by a gene deletion. Thus, stick17 and stick18 do not display any residual PINCH activity that ameliorates the mutant phenotype relative to what is observed in a molecular null. Moreover, neither stick17 nor stick18 heterozygotes display any cellular defects or loss of viability (data not shown) that might be anticipated if the stick17 and stick18 alleles produced a dominant-negative product.

Because pinch transcripts are maternally inherited, we evaluated the phenotype of animals in which both zygotically and maternally derived PINCH were eliminated by construction of germline clones. Analysis of maternal/zygotic stick mutants did not reveal additional phenotypes that were not evident in zygotic stick mutants; however, the disturbance in muscle morphology was evident at an earlier stage than for the zygotic mutants, with actin clumping apparent in some muscle cells by the end of stage 16 (data not shown), consistent with the time of onset of muscle contraction.

**PINCH protein is prominently expressed in embryonic muscle where it localizes at muscle-attachment sites**

Since the stick mutants exhibited defects in the anchorage of actin filaments at the myotendinuous junction, we postulated that PINCH might be a constituent of these cell-substratum attachment sites. Indeed, by immunocytochemical analysis, we detect PINCH protein in the developing somatic muscles, with prominent enrichment at the muscle-attachment sites (Fig. 3A-C). PINCH is also detected in other musculatures including the dorsal vessel (the heart equivalent in Drosophila; Fig. 3A,C), the visceral musculature surrounding the gut (Fig. 3B,D) and in the pharyngeal muscles (Fig. 3B,C). There is also prominent staining in the midgut epithelium (Fig. 3D). The affinity-purified serum also labels the chordotonal organs, but this appears to be due to crossreaction with another protein because this staining remains in stick17 maternal/zygotic mutants, whereas all muscle attachment site staining is absent (data not shown).

**Integrins are necessary for the proper localization of PINCH to the muscle-attachment sites**

The Drosophila integrin subunits αPS2 and βPS are also enriched at muscle-attachment sites, where they participate in the adhesion of the muscle termini to a specialized ECM, the tendon cell matrix (Leptin et al., 1989; Brown, 1994). Using confocal microscopy, we found that PINCH is precisely colocalized with βPS integrin at muscle-attachment sites in the muscle-attachment sites.
somatic muscle termini (Fig. 4A,B). PINCH and βPS integrin proteins also display overlapping patterns of concentration in other tissues such as the visceral musculature, pharyngeal muscles and epithelial tissues (data not shown).

Given the striking accumulation of PINCH and βPS integrin at muscle-attachment sites, we tested whether PINCH depends on integrins to become properly distributed in the muscle. PINCH protein distribution was examined in embryos harboring null alleles of either βPS integrin (myospheroid) or αPS2 integrin (inflated). The αPS2βPS heterodimer is the integrin complex present on the muscle side of the myotendinous junction, and loss of either subunit prevents the localization of the other subunit (Leptin et al., 1989). Compared with wild-type embryos in which PINCH displays a striking localization at muscle-attachment sites (arrow, Fig. 4C), PINCH was not enriched at the muscle termini of myospheroid (Fig. 4D,E) or inflated (data not shown) mutants. The lack of PINCH staining in the myospheroid and inflated mutant embryos was not due to a failure in antibody penetration or disintegration of the muscle-attachment sites, as the nonspecific immunoreactivity of the chordotonal organs was still present (Fig. 4E), and the cytoskeletal protein PAK remains robustly localized at residual muscle-attachment sites in myospheroid embryos (Fig. 4F) and in inflated embryos (Bloor and Kiehart, 2001). The presence of PAK at the muscle borders in myospheroid mutants provides support for the conclusion that the loss of PINCH from muscle attachments in an integrin mutant is not due to a general defect in these junctions, and instead indicates a direct dependence of PINCH on integrins for its distribution in mature muscle. We do detect some PINCH protein concentrated at the muscle termini in younger myospheroid embryos (data not shown). The most straightforward interpretation of these results is that PINCH requires integrins for its maintenance at muscle attachments, and not for its initial localization. However, although several groups have failed to detect maternally supplied βPS integrin in myospheroid embryos at this time of development (Bunch et al., 1992) (data not shown), it remains formally possible that some residual βPS protein is present to recruit PINCH to the junctional complex at this earlier stage. In any case, our findings illustrate that, at a minimum, integrins are required for maintenance of PINCH at the junctional complexes.

In complementary experiments, we examined βPS integrin distribution in wild-type and stick mutant embryos. Wild-type embryos show a striking accumulation of βPS integrin at muscle-attachment sites (Fig. 4G) (Leptin et al., 1989). Although muscle morphology is perturbed in stick mutants, βPS retains the capacity to localize at muscle-attachment sites when PINCH function is compromised by mutation (Fig. 4H). Thus, the appropriate targeting of βPS integrin to the cell surface and their concentration at adhesive junctions can occur in the absence of PINCH.

**Fig. 4.** PINCH is dependent on integrins for its enrichment at muscle-attachment sites. (A,B) Optical section through a stage 16 embryo, showing localization of the indicated proteins at the muscle-attachment sites. (A) PINCH immunoreactivity. (B) βPS integrin immunoreactivity. The merge of the boxed regions in the stained embryos is shown in the lower corner of the panel. (C,D) Optical sections near the lateral surface of stage 16 embryos stained for PINCH. (C) PINCH enrichment at muscle-attachment sites in wild-type muscle cells. (D) PINCH distribution in myospheroid mutant muscle cells. Note lack of enrichment at the muscle termini (arrows). (E,F) Lateral views of stage 16 myospheroid embryos. (E) PINCH distribution. (F) Pak distribution. Pak remains prominently enriched at muscle-attachment sites (arrows in F), while PINCH is diffuse. Arrowheads in E indicate background immunoreactivity against chordotonal organs present in the affinity-purified PINCH antiserum. (G,H) Ventral views of stage 16 embryos stained with a monoclonal antibody against βPS integrin. (G) βPS integrin distribution in a wild-type embryo. (H) βPS integrin distribution in a stick18/1(3)097 embryo. βPS integrin remains enriched at the muscle-attachment sites, indicating that functional PINCH is not required for integrin localization to the myotendinous junction.
The lethal phenotype associated with stck mutations does not arise due to a failure of ILK to localize properly

Based on biochemical studies in vertebrate systems, it has been suggested that an integrin-ILK-PINCH complex might be necessary for integrin-dependent cell adhesion (Li et al., 1999; Tu et al., 1999; Wu, 1999). Consistent with this view, a recent characterization of Drosophila ILK revealed that ILK colocalizes with βPS integrin at muscle-attachment sites and is required for integrin function (Zervas et al., 2001). As can be seen in Fig. 5A-C, PINCH and ILK display completely overlapping patterns of localization in Drosophila muscle, with both proteins prominently enriched at the muscle-attachment sites. PINCH and ILK are also co-expressed in the visceral mesoderm and pharyngeal muscles. Thus, PINCH, ILK and βPS integrin are co-residents of the same cellular compartments in vivo.

To test directly whether PINCH is present in a molecular complex with ILK in vivo, we performed native immunoprecipitation studies with embryo extracts prepared from a transgenic line carrying an ILK::GFP genomic construct, that was previously shown to maintain wild-type ILK activity based on its ability rescue the ilk mutant phenotype (Zervas et al., 2001). In the anti-PINCH immunoprecipitate, a band of the expected size of ~75 kDa for the ILK-GFP protein is detected with a mAb against GFP (Fig. 5D, lane 1). This band was absent in anti-PINCH immunoprecipitates from wild-type embryos (Fig. 5D, lanes 2 and 4) nor an antiserum served as negative controls, while PINCH immunocomplexes were isolated with the PINCH preimmune serum (PI) (C) Merged image of A,B. (D) Native immunoprecipitation of PINCH and associated proteins from Drosophila embryonic lysates. Lanes 1-4 represent different immunoprecipitation experiments, run out on an SDS-PAGE gel. The resulting blot was probed with the antisera indicated on the right-hand side. Immunoprecipitations with the PINCH preimmune serum (PI) serve as negative controls, while PINCH immunocomplexes were isolated with the affinity-purified PINCH antiserum (anti-Pin). (E,F) ILK::GFP distribution in a wildtype (E) and stck17 germline/zygotic mutant (F) stage 16 embryo. The ILK fusion protein is still concentrated at the muscle-attachment sites in the stck mutant embryo (arrows in F).
Fig. 6. PINCH function is essential for adhesion between wing epithelial sheets. (A) Production of stck$^{17}$ homozygous clones in wing tissue leads to blister formation. (B-D) Confocal micrographs of a developing wing disc (~45 hours after puparium formation). (B) βPS integrin protein is enriched at the basal junctions between the two epithelial layers (arrow). (C) PINCH protein is also expressed in the developing wing, and displays a similar subcellular distribution (arrow). (D) Merge of B and C.

associated with wing cell membranes; βPS integrin displays a similar pattern at this stage of development (data not shown). Later in development, when the wing epithelia have become apposed, βPS integrin becomes enriched at basal junctions that form between the two layers (Fristrom et al., 1993) (Fig. 6B). We find that PINCH is enriched at this junction and is also associated with the cell cortex coincident with sites of integrin accumulation (Fig. 6C,D). Collectively, our findings support the view that PINCH is required for integrin function in both embryos and adults.

**DISCUSSION**

Interest in PINCH has increased in the last few years due to its potential role as an integrin effector molecule (Tu et al., 1998; Li et al., 1999; Wu, 1999). Our genetic analysis of PINCH function has led to four main conclusions: (1) *Drosophila* PINCH is encoded by the stck locus and is essential for embryonic development and maintenance of tissue architecture; (2) PINCH is necessary for stable actin-membrane anchorage in muscle and contributes to integrin-dependent adhesion in muscle cells and epithelial cells; (3) integrins are required for the stable association of PINCH with muscle-attachment sites; and (4) the lethal stck mutant phenotype cannot be attributed to mislocalization of the PINCH-binding partner, ILK, whose recruitment to muscle-attachment sites appears normal in stck mutant embryos.

**A link between PINCH and integrin function**

Genetic analyses of the roles of integrins in *Drosophila* have clearly highlighted the importance of integrins for adhesion and signaling in vivo (Martin-Bermudo and Brown, 1999; Bokel and Brown, 2002). We report that *Drosophila* PINCH is colocalized with integrins in both muscle and epithelial cells. Integrins retain the capacity to accumulate at muscle-attachment sites in stck mutants, illustrating that PINCH does not have an obligatory role in the proper processing and membrane targeting of integrins in vivo. The integrin staining in stck mutants does lack the high degree of order and lateral registration observed in wild-type embryos. In the *Drosophila* system, it is difficult to distinguish whether this modest disorganization simply reflects the underlying disturbance of the musculature or if it is revealing some contribution of PINCH to maintenance of spatially restricted integrin localization. In *C. elegans* embryos in which PINCH function is compromised by unc-97 mutation, both integrin and vinculin spread laterally beyond their normal zones of accumulation in dense plaques, suggesting a role for PINCH in clustering of adhesive junction components in this system (Hobert et al., 1999).

Interestingly, PINCH depends on the presence of integrins for its stable accumulation at muscle-attachment sites. The physiological roles of several other proteins, including Talin, ILK, Myosin II and Short Stop, that colocalize with βPS integrin at *Drosophila* muscle-attachment sites have recently been characterized (Gregory and Brown, 1998; Bloor and Kiehart, 2001; Zervas et al., 2001; Brown et al., 2002). These proteins display variable levels of dependence on integrins for their localization. Like Talin, a well-established integrin effector (Horwitz et al., 1986; Brown et al., 2002; Calderwood et al., 2002), PINCH depends on the presence of integrins for its concentration at muscle-attachment sites. The reliance of PINCH and Talin on integrins for their spatially restricted accumulation in muscle emphasizes their connection to the integrin receptors.

Integrins must establish links to both extracellular determinants and to intracellular cytoskeletal elements in order to support strong adhesion (Crichtley et al., 1999; Brown et al., 2000). Examination of the cellular defects in stck mutant muscle suggests that PINCH contributes to the stabilization of actin-membrane linkages at integrin-rich adhesion sites. In a stck mutant muscle cell, the actin filaments lose their linear organization and eventually accumulate in clumps at one end of the cell. We interpret these defects to mean that a primary consequence of disturbed PINCH function is a destabilization of the linkage between the actin cytoskeleton and the muscle membrane; it appears that the actin-membrane attachments in stck mutants lack the mechanical strength to remain intact during cyclic muscle contraction. Because integrin functionality relies on the ability of the receptors to establish a transmembrane link between the cytoskeletal elements and the extracellular matrix, reduced substratum attachment strength and/or stability might also be expected to occur if membrane cytoskeletal linkages were compromised. Consistent with this prediction, loss of adhesion is evident in...
the stick17–/– wing cell clones and, to some extent, in muscles of stck mutant embryos.

**The relationship between PINCH and integrin-linked kinase**

The molecular architecture of PINCH suggests that it may function as a platform for the docking and/or productive juxtaposition of protein partners. ILK, a binding partner of PINCH, is thus a candidate to collaborate with PINCH in the stabilization of integrin-cytoskeletal linkages. Consistent with the view that PINCH and ILK could cooperate to promote stable actin anchorage at sites of integrin-mediated adhesion, the phenotypes that result from compromised function of either protein in *Drosophila* are very similar (this report) (Zervas et al., 2001). Moreover, we show that PINCH and ILK are colocalized in *Drosophila* embryos and are recovered in a protein complex isolated from embryos by immunoprecipitation. *Drosophila* PINCH also interacts directly with ILK using two-hybrid methods (J. L. Kadrmas, S. M. Pronovost and M.C.B., unpublished). These latter results are consistent with findings reported previously for vertebrate PINCH and ILK (Li et al., 1999; Tu et al., 1999). Confirmation that PINCH and ILK interact in *Drosophila* was important as biochemical findings in vertebrate systems are not always recapitated in the fly (Zervas et al., 2001). PINCH and ILK also colocalize at actin-membrane anchorage sites in *C. elegans* muscle, and elimination of either gene product was shown to produce a paralyzed at twofold stage (PAT) phenotype similar to that seen for β-integrin mutants (Hobert et al., 1999; Mackinnon et al., 2002). Collectively, results in both invertebrate and vertebrate systems illustrate that the capacity to form a PINCH/ILK complex has been conserved through evolution.

Given the fact that ILK and PINCH colocalize, co-precipitate and have similar loss of function phenotypes, it was possible that disturbed PINCH function could adversely affect ILK localization and that such mislocalization might account for the stck mutant phenotype. To explore this possibility we examined the localization of ILK in stck mutant embryos and found that ILK is unperturbed in its ability to accumulate at muscle-attachment sites, even when a dramatic lethal phenotype is evident in stck mutant embryos. As noted above, βPS integrin also accumulates at muscle-attachment sites in stck mutant embryos. These findings illustrate that the proper localization of integrin and ILK is not sufficient to stabilize actin membrane linkages at sites of integrin-dependent adhesion, and define PINCH as a critical component of the molecular machinery necessary for the tethering of actin to the integrin-rich membranes.

The demonstration that single ilk and stck mutants both display deficiencies in integrin-dependent processes illustrates that neither PINCH nor ILK is sufficient on its own to support full integrin function. It is possible that PINCH acts as a positive regulator of ILK function, either by modulating ILK function by direct binding or by recruitment of an ILK-modifying factor. Alternatively, ILK may activate some PINCH function that is crucial for stabilization of actin-membrane linkages. Finally, a PINCH-ILK protein complex may be a key component of the platform necessary for the recruitment of other proteins required to achieve stable actin-membrane associations. In this regard, it is of interest that PINCH and ILK can be recovered in a complex with the ILK-binding partner, CH-ILKBP, a calponin domain-containing protein related to Affixin and Actopaxin that could provide the link to actin filaments (Tu et al., 2001; Yamaji et al., 2001; Nikolopoulos and Turner, 2002). Because the localization of *Drosophila* PINCH is dependent on integrins, the establishment of PINCH-ILK complexes at muscle-attachment sites would not be supported in the absence of integrin function. This dependence of PINCH localization on integrins could provide a means to couple integrin adhesive function to its role in cytoskeletal anchorage.

In vertebrate cells, PINCH and ILK appear to be mutually dependent on each other for their localization to integrin-rich focal adhesions (Zhang et al., 2002b). However, as noted above, despite their ability to interact with each other, PINCH and ILK show distinct requirements for their recruitment to specific subcellular domains in *Drosophila*. In particular, we show that PINCH requires functional integrins for its localization to muscle-attachment sites, whereas it has previously been demonstrated that *Drosophila* ILK fails to bind integrins directly and localizes normally in an integrin mutant (Zervas et al., 2001). Rather than employing an association with integrins, ILK may rely on a protein such as Paxillin for its targeting to integrin-rich sites (Nikolopoulos and Turner, 2001). Although *Drosophila* PINCH requires integrins for its stable accumulation at muscle-attachment sites, there is no evidence that PINCH can associate directly with integrin cytoplasmic domains, therefore additional proteins probably act as a bridge.

**Drosophila as a model system for the study of integrin function**

Recently, two laboratories have independently conducted a clever genetic screen for potential integrin effectors in *Drosophila* (Prout et al., 1997; Walsh and Brown, 1998). These screens relied on the fact that loss of integrin function results in a readily scorable blistering phenotype because of compromised epithelial cell adhesion in the wing. The screening strategy employed mitotic recombination to allow examination of homozygous mutant cell clones in an otherwise heterozygous background. This approach permitted a large number of independent mutations to be examined for effects on integrin-dependent adhesion. Over 25 loci were identified that could produce wing blisters when mutated. However, to date, the molecular lesions associated with these wing blister mutations have only been identified for a few loci (Prout et al., 1997; Gregory and Brown, 1998; Walsh and Brown, 1998; Brown et al., 2002). The identification of *stck* as the PINCH gene in *Drosophila* gives additional confidence that the genetic screens are identifying molecules important for integrin function. Moreover, the unique, but related, phenotypes that result when genes encoding different components of integrin adhesive membranes are mutated have provided significant new insight into how various accessory proteins cooperate with integrin. For example, Short Stop appears to contribute to integrin-dependent cell adhesion by coupling the microtubule cytoskeleton to the adhesive membrane (Gregory and Brown, 1998). Our characterization of PINCH loss of function phenotypes suggests that PINCH plays a key role in stabilizing the link between cytoplasmic actin filaments and the integrin-rich adhesive membrane. Collectively, these analyses allow a precise molecular dissection of integrin function in *Drosophila*.
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