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blistery encodes *Drosophila* tensin protein and interacts with integrin and the JNK signaling pathway during wing development

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SUMMARY

Tensin is an actin-binding protein that is localized in focal adhesions. At focal adhesion sites, tensin participates in the protein complex that establishes transmembrane linkage between the extracellular matrix and cytoskeletal actin filaments. Even though there have been many studies on tensin as an adaptor protein, the role of tensin during development has not yet been clearly elucidated. Thus, this study was designed to dissect the developmental role of tensin by isolating *Drosophila* tensin mutants and characterizing its role in wing development. The *Drosophila* tensin loss-of-function mutations resulted in the formation of blisters in the wings, which was due to a defective wing unfolding process. Interestingly, by^1-the mutant allele of the gene *blistery* (*by*)-also showed a blistered wing phenotype, but failed to complement the wing blister phenotype of the *Drosophila* tensin mutants, and contains Y62N/T163R point mutations in *Drosophila* tensin coding sequences. These results demonstrate that *by* encodes *Drosophila* tensin protein and that the *Drosophila* tensin mutants are alleles of *by*. Using a genetic approach, we have demonstrated that tensin interacts with integrin and also with the components of the JNK signaling pathway during wing development; overexpression of *by* in wing imaginal discs significantly increased JNK activity and induced apoptotic cell death. Collectively, our data suggest that tensin relays signals from the extracellular matrix to the cytoskeleton through interaction with integrin, and through the modulation of the JNK signal transduction pathway during *Drosophila* wing development.

Key words: *blistery*, *Drosophila*, Integrin, JNK, Tensin, Wing blister

INTRODUCTION

Tensin is a focal adhesion molecule that binds to actin filaments through its N terminus (Lo et al., 1994a; Lo et al., 1994b; Chuang et al., 1995). In addition, it contains two functional motifs, including a Src homology domain 2 (SH2) and a phosphotyrosine binding (PTB) domain (Davis et al., 1991; Chen et al., 2000; Chen et al., 2002). This conserved domain structure gives significant clues to its possible function, including potential roles in cell signaling.

Tensin is best known as an adaptor protein linking integrin to the actin cytoskeleton. Integrins are a family of the transmembrane receptors that are localized in focal adhesions (Hynes, 1992). The extracellular domain of integrin interacts with the extracellular matrix, and its cytoplasmic domain anchors actin filaments to the plasma membrane through the focal adhesion protein complex (Burridge et al., 1988; Hynes, 1992; Lo et al., 1994a; Jockusch et al., 1995). Integrin is also believed to participate in diverse biological events such as cytoskeletal restructuring, cell motility and even cell survival via focal adhesion complexes that include tensin, focal adhesion kinase (FAK), Src kinase and protein kinase C (Burridge et al., 1988; Schwartz et al., 1995; Giancotti and Ruoslahti, 1999). Most interestingly, integrin is involved in various cell signaling pathways through its association with focal adhesion proteins. For example, integrin activates ERK-MAP kinase by promoting the SH2 domain-mediated association of Grb2 with tyrosine kinases such as FAK and c-Src in focal adhesions (Schlaepfer et al., 1994). The phosphoinositide 3 kinase (PI3K)-dependent signaling pathway is also activated by integrin through FAK-dependent mechanism (Chen and Guan, 1994; Shaw et al., 1997; Reiske et al., 1999). In addition, several reports have demonstrated that JNK is also activated by integrins when cells are attached to the extracellular matrix (Miranti et al., 1998; Oktay et al., 1999).

The role of tensin as an adaptor for integrin and as a required component in focal adhesions suggests the possibility that it may act as a mediator of integrin signaling. In support of this idea, much indirect and direct evidence has been collected. Previously, the SH2 and PTB domains in the C terminus of tensin have been demonstrated to bind tyrosine phosphorylated proteins such as PI3K and p130 CAS (Salgia et al., 1995; Salgia et al., 1996; Auger et al., 1996). In addition, tensin itself is phosphorylated at serine, threonine and tyrosine residues when cells are stimulated by either cell adhesion (Bockholt and

Burridge, 1993), growth factors (Jiang et al., 1996) or oncogenes, including v-Src and Bcr/Abl (Davis et al., 1991; Salgia et al., 1995). Indeed, a recent study has shown that overexpression of tensin alone can activate JNK in human embryonic kidney 293T cells (Katz et al., 2000). According to this report, the tensin-mediated JNK activities are independent of the activities of small GTP-binding proteins such as Rac and Cdc42, but dependent on the activity of SEK (Katz et al., 2000).

In the present study, we have shown that *by*, one of the previously reported genes to result in a blistered wing phenotype when disrupted, encodes the *Drosophila* ortholog of mammalian tensins, and with the *by* mutants, we were able to characterize the functions of tensin in vivo. The blistered wing phenotype of the *by* loss-of-function flies was demonstrated to be caused by a defective wing unfolding process after eclosion. Additionally, using a genetic approach and immunohistochemistry, we have shown that tensin functionally interacts with integrin and the JNK signaling pathway. Our results demonstrate the in vivo roles of tensin in development and suggest that tensin might be a transducer of signals from integrin to the JNK signaling pathway.

MATERIALS AND METHODS

Drosophila strains

The Upstream Activation Sequence (UAS) fly lines of *basket* (*bsk*; *Drosophila JNK*), *hemipterous* (*hep*; *Drosophila MKK7*) and *rolled*^{Sem} (*rl*^{Sem}; a gain-of-function allele of *Drosophila ERK*) were gifts from Drs M. Mlodzik (EMBL-Heidelberg, Germany), T. Adachi-Yamada (Kobe University, Japan) and D. Bohmann (University of Rochester, USA), respectively. *hep*^{1} fly line was kindly provided by Dr S. Noselli (CNRS, France). The fly lines *if*^{3}, *bsk*^{1} and *by*^{1} were obtained from the Bloomington Stock Center (Bloomington, IN). MS1096-GAL4 driver line was a gift from Dr M. Freeman (MRC Laboratory of Molecular Biology, UK).

The *blistery* mutants

The *blistery* mutant *by*^{2} is a P-element insertion line. The *by*^{2} line was isolated accidentally during the generation of UAS lines of a *Drosophila* EST clone, SD01679. Of many UAS lines generated, this line was distinct from others, showing a blistered wing phenotype and partial sterility for the females. At first, these features made us speculate that they were caused by the inserted transgene, but as other transgenic lines of the EST inserted at other loci did not display the same phenotype, and the imprecise excision lines also showed the same phenotype as their original line, we concluded that the phenotype was the outcome of a gene disruption caused by the inserted P-element. The identity of the disrupted gene was revealed by performing inverse-PCR and sequencing the regions flanking the P-element. The P-element insertion site in this line was 28 bp upstream of the *by* open reading frame (ORF), which encodes *Drosophila* tensin protein, and this mutant line was thus named *by*^{2}. P-element excision alleles were generated as Horowitz and Berg performed previously (Horowitz and Berg, 1995), except that TMSΔ2-3 was used as the transposase source and *white* was a background mutation. The molecular characterization of each mutant line was performed by genomic DNA polymerase chain reactions (PCR). We generated six revertants and two excision alleles of *by*^{2} from a P-element excision experiment. In *by*^{ex49}, one of the imprecise excision alleles, 3.56 kb of genomic region including most of *by* exon was deleted, and in the other imprecise excision line, *by*^{ex10}, 1.1 kb fragment of the original P-element remained in the 5′ upstream of the *by* locus (Fig. 3A).

Construction of pUAST-*by* plasmids and generation of transgenic fly lines

Full-length and partially deleted *by* genes (ΔN: amino acids 1-450 deleted, ΔC: amino acids 451-720 deleted, ΔPTB: amino acids 567-720 deleted) were each subcloned into separate pUAST vectors. Standard procedures for the egg injection were used for the transformation of *Drosophila* (Ashburner, 1989), using a microinjector model IM300 (Narishige, Japan) and an Axiovert 25 micromanipulator (Carl Zeiss, Germany).

Northern blot analyses

Total RNA, extracted by the easy-Blue^{TM} system (Intron, Korea), was separated by electrophoresis on denaturing formaldehyde agarose gels in MOPS buffer, transferred onto a nylon membrane, and successively hybridized with nick-translated ^{32}P-labeled cDNA probe. The probe was made with the PCR product that corresponds to the 3′ region of the *by* amplified with the primers (5′GGTGTCCGCCGATTCCG-TACAATTT3′ for 5′ and 5′GGCCCGCACCGGTGGTAC3′ for 3′). Hybridized probes were visualized by autoradiography.

RT-PCR and RNA in situ hybridizations

For RT-PCR, total RNA was isolated from wild-type and the *by* mutant adult flies using the easy-Blue^{TM} system (Intron, Korea), and reverse transcribed into cDNA. PCR was performed with the *by* primers. One set of PCR primers (5′ATGCGGACGCCGTACGAAG-AAAG3′ for 5′ and 5′CACATTCGTATTCGTTGGCC3′ for 3′) was designed to amplify the 5′ portion of *by* (designated as ‘a’ in Fig. 6A), and the other set (5′ATGCGTCACTTCCTCATCGAG3′ for 5′ and 5′CGAGGAGACCTTGAAGTGCAC3′ for 3′) was prepared to amplify the 3′ region of *by* (designated as ‘b’ in Fig. 6A).

In situ hybridization experiments were performed with a digoxigenin-labeled RNA probe corresponding to the ‘b’ region of *by* (Fig. 6A), as previously described (Cho et al., 2001).

Histochemical analyses

Drosophila wing imaginal discs were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). They were then washed in PBST (PBS + 0.1% Tween 20) and blocked in PBST with 3% bovine serum albumin. The samples were incubated overnight at 4°C with either anti-beta PS integrin mouse antibody [1:200 dilution; a gift from Dr D. L. Brower (University of Arizona, Tucson, AZ)] or anti-phosphospecific JNK rabbit antibody (1:200 dilution; Promega, WI). Next, the samples were further incubated for 4 hours at room temperature in either FITC-labeled anti-mouse secondary antibody (1:200 dilution; Sigma, MO) or HRP-conjugated anti-rabbit IgG secondary antibody (1:200 dilution; Molecular Probes, OR), and analyzed using an LSM510 laser confocal microscope (Carl Zeiss, Germany). Alexa Fluor 568 tyramide (Molecular Probes, OR) was used as a substrate for HRP-conjugated anti-rabbit IgG secondary antibody. In order to visualize the actin structure, pupal wing discs were stained with phalloidin-TRITC (Sigma, MO) for 20 minutes. For Acridine Orange (Sigma, MO) staining, third-instar larval wing discs were dissected in PBS and incubated for 5 minutes in 1.6 μM Acridine Orange-PBS solution. The samples were observed by a confocal microscope (Carl Zeiss, Germany).

RESULTS

Characterization of *Drosophila* tensin and its mutants

The sole *Drosophila* ortholog of mammalian tensins, CG9379 (GenBank accession number: NM_141644, Protein_ID: NP_649901), was identified in the *Drosophila* genome data bank by BLAST search, and corresponds to the open reading frame deduced from *Drosophila* EST clone, RH56077 (GenBank accession number: AY094941) (Fig. 1A). *Drosophila*

tensin has a predicted molecular weight of 79 kDa, with a much shorter N-terminal region than that of mammalian counterpart. Although the N-terminal sequence of tensin is not conserved, its C-terminal region of about 350 amino acids, which includes the SH2 and PTB domains, exhibits significant homology to the human homologs, with about 40% amino acid identity (Fig. 1B).

To determine the expression stage and transcript complexity of *Drosophila* tensin, we performed Northern blot analyses at various developmental stages using a probe derived from the *Drosophila* tensin cDNA (see Materials and Methods). A single transcript of about 3.0 kb was detected throughout all the developmental stages (Fig. 1C).

Next, we decided to investigate the in vivo function of *Drosophila* tensin using genetic approaches. As described in the Materials and Methods, we found one P-element insertion line, by^2, containing a P-element in the 5′ flanking sequence of the *Drosophila* tensin open reading frame (Fig. 1A). To examine whether the P-element insertion in by^2 hampers the transcription of *Drosophila* tensin, we performed RT-PCR, which showed highly reduced *Drosophila* tensin expression level in the whole body (Fig. 3C). In addition to by^2, six revertants and two imprecise excision alleles of by^2 were generated as described in the Materials and Methods. These newly obtained mutants were verified by genomic PCR and subsequent sequencing analyses. The expression levels of *Drosophila* tensin in these lines were also examined by RT-PCR. The transcript levels of *Drosophila* tensin were completely normal in the revertant lines (data not shown) compared with wild type. By contrast, *Drosophila* tensin expression was highly reduced or completely missing in the imprecise excision alleles (Fig. 3C).

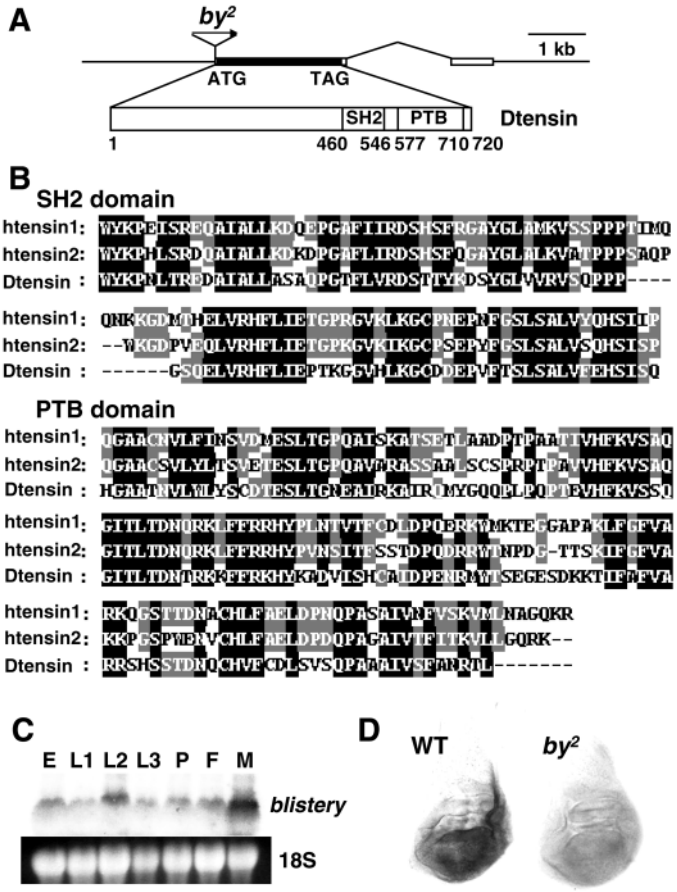

Fig. 1. Characterization of *by*. (A) Genomic structure of *by* and the corresponding protein domain structure with the amino acid number presented underneath. The P-element insertion site of the by^2 mutant allele is noted. (B) *Drosophila* tensin (Dtensin) exhibits highly homologous C-terminal SH2 and PTB domain structures to its human orthologs. (C) *by* mRNA was expressed throughout all the developmental stages, and its level was highest in the adult male (upper panel). 18S rRNA was used as a loading control (lower panel). E, Embryo; L1, first instar larva; L2, second instar larva; L3, third instar larva; P, pupa; F, female; M, male. (D) In RNA in situ hybridization analyses, *by* was abundantly expressed in the wing pouch of wild-type flies (left), while significantly reduced signal was detected in the wing imaginal disc of by^2 mutant (right).

Drosophila tensin mutants display a blistered wing phenotype

Homozygous but not heterozygous by^2 mutant flies showed blisters in their wings (Fig. 2A). To see whether this phenotype is related to *Drosophila* tensin expression, in situ hybridization was performed in wing imaginal discs. As shown in Fig. 1D, *Drosophila* tensin expression was dramatically reduced in the by^2 mutants (Fig. 1D, right panel) compared with the control (Fig. 1D, left panel). Interestingly, the *Drosophila* tensin transcript was highly enriched in the wing pouch (Fig. 1D, left panel). We confirmed that the blistered wing phenotype was indeed caused by a deficiency in *Drosophila* tensin expression, by generating transgenic flies specifically overexpressing *Drosophila* tensin within a homozygotic by^2 genetic background. Ectopic expression of full-length *Drosophila* tensin in wing imaginal discs dramatically rescued the wing blister phenotype of by^2 (Fig. 2B). Moreover, two additional *Drosophila* tensin loss-of-function alleles, by^{ex10} (Fig. 2C) and by^{ex49} (data not shown) failed to complement the blistered wing phenotype of the by^2 mutants. However, by^{rv8}, one of the revertants, fully complemented the by^2 mutation (Fig. 2D). These results unequivocally demonstrate that the blistered wing phenotype in the *Drosophila* tensin mutants is due to a defect in *Drosophila* tensin function.

Drosophila tensin mutants are new alleles of *by*

We searched all the reported mutant lines with a blistered wing phenotype that mapped around the cytogenetic location of *Drosophila* tensin, 85D22, and found one mutant line, $blistery^1$ (by^1) – the loss-of-function allele of the *blistery* (*by*) gene. The mutation site of by^1 has been reported to be located around 85D, but specific details have not yet been determined. The by^1 mutants display blisters in the wing subterminal region (Glass et al., 1934; Prout et al., 1997; Walsh and Brown, 1998), which is identical to the phenotype of the *Drosophila* tensin mutants such as by^2 (Fig. 2A) and by^{ex49} (data not shown).

Significantly, the transheterozygotic alleles of by^1 and by^2 failed to complement each other's blistered wing phenotype (Fig. 2F), implying that they are alleles of the same gene. Consistently, by^{ex10} and by^{ex49} also failed to complement the blistered wing phenotype of by^1 (data not shown).

To further confirm that the *Drosophila* tensin mutants are allelic to *by*, we sequenced the *Drosophila* tensin locus in the by^1 mutants. As expected, we found that by^1 allele contains two missense point mutations that convert tyrosine 62 and

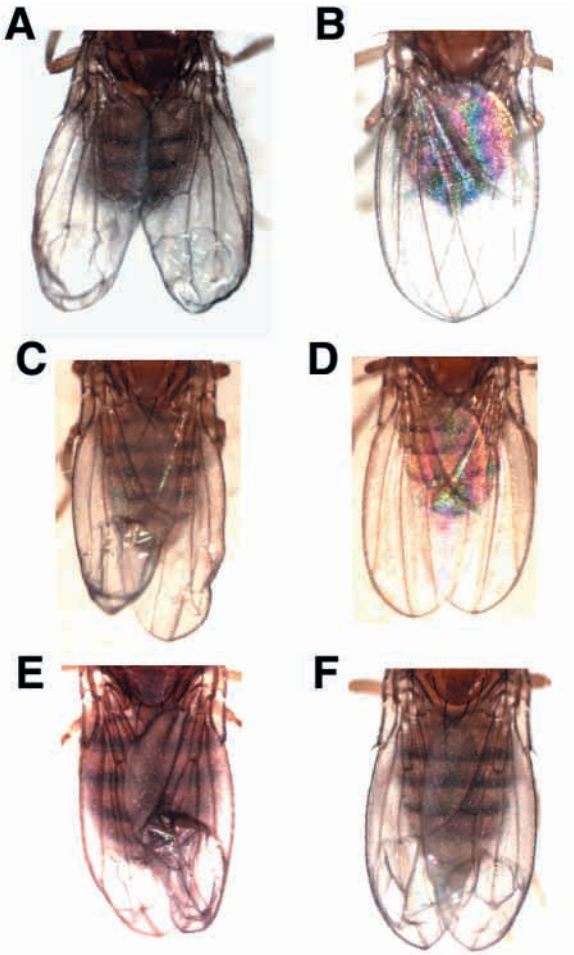

Fig. 2. The blistered wing phenotype of various *by* mutants. (A) by^2/by^2. (B) MS1096/X; by^2, UAS-*by*/by^2. (C) by^2/by^{ex10}. (D) by^2/by^{rv8}. (E) by^1/by^1. (F) by^1/by^2.

threonine 163 to asparagine (Y62N) and arginine (T163R) in tensin, respectively (Fig. 3A). Therefore, we conclude that our *Drosophila* tensin mutants and by^1 are different alleles of *by*, and hereafter we refer to *Drosophila* tensin as *by*.

The severity of the blistered wing phenotype varies depending on *by* expression levels

The *by* mutant flies exhibited a blistered wing phenotype with varying degrees of severity, which can be classified into three groups; normal wing, class I wing which contains a small blister (< 1/4 size of the total wing area) within a restricted region, and class II wing with a large blister (> 1/4 size of the total wing area) resulting in a crumpled wing phenotype (Fig. 3B). We determined the severity of the wing blister phenotype by measuring the frequency of each wing class. By comparing the severity in various mutants (Fig. 3B) and their *by* expression levels (Fig. 3C), we found that the severity of wing blisters is closely related to the level of the *by* transcript. For example, a relatively mild phenotype of the by^{ex10} mutants can be explained by higher expression of *by* than the other *by* mutants.

Besides the blistered wing phenotype, the *by* mutants exhibited reduced hatching rate of laid eggs (Fig. 3D). The hatching rate of the mutant eggs was reduced to about 60% of wild type, while the survival rate of mutants after hatching was not significantly affected (Fig. 3D). These results demonstrate that tensin plays important roles during *Drosophila* early development as well. Our unpublished data suggest that the impairment in egg hatching is resulted from defective fertilization, therefore the *by* mutants are homozygous viable with decreased fertility.

The blistered wing phenotype is resulted from a defective wing unfolding process after eclosion

Drosophila wing development after pupariation (AP) consists of two distinct stages: prepupal and pupal wing morphogenesis (Fristrom et al., 1993; Fristrom et al., 1994). And pupal wing

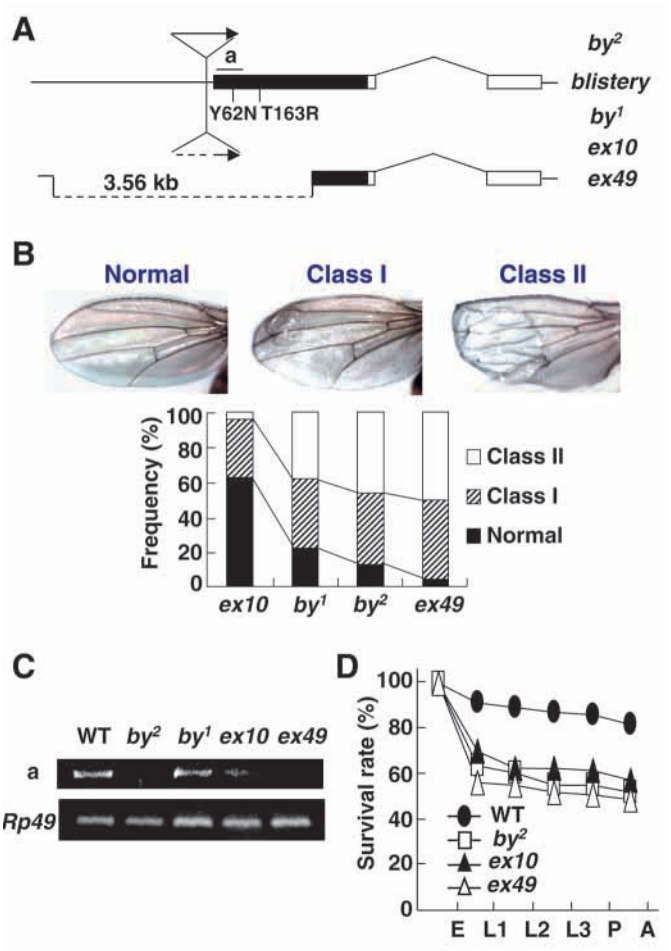

Fig. 3. Characterization of the *by* mutant phenotype. (A) A schematic drawing of the *by* genomic locus and the genomic structures of the *by* mutant alleles. *ex10*, by^{ex10}; *ex49*, by^{ex49}. (B) Comparison of the severity of the blistered wing phenotype between different *by* mutants. (C) Determination of expression levels of *by* transcript in various *by* mutant alleles using RT-PCR method. 'a' represents 5′ region of the *by* gene (shown in A). Ribosomal protein 49 (*Rp49*) was used as an internal control. RT products were amplified 28 times to detect 'a' region, and 25 times to detect *Rp49* levels in PCR reactions. (D) The *by* loss-of-function mutants exhibited decreased hatching rates from eggs. Black circles and triangles represent wild type and by^{ex10}, respectively. White squares and triangles represent by^2 and by^{ex49}, respectively. E, egg; L1, first instar larva; L2, second instar larva; L3, third instar larva; P, pupa; A, adult.

morphogenesis is further divided into three stages: separation (11-12 hours AP) of the ventral cell layer from the dorsal layer, re-apposition of the inter-vein cells (21-36 hours AP) and re-separation (60 hours AP) of the two cell layers. Shortly after eclosion, wings expand and unfold by an influx of hemolymph. According to a previous report, PS integrins are required for the attachment of the two wing surfaces during pupal wing re-apposition and for the maintenance of the wing bilayer (Brabant et al., 1996).

To determine the detailed roles of tensin during wing morphogenesis, we examined the pupal wings of the by^2 flies. As shown in Fig. 4, we were not able to observe any differences in the attachment of two wing surfaces (Fig. 4A-H) and in the integrin localization (Fig. 4I-L) between wild type (Fig. 4, left panels) and by^2 (Fig. 4, right panels) wings during both prepupal apposition (4-6 hours AP, Fig. 4A-D) and pupal reapposition stages (30-36 hours AP, Fig. 4E-L).

Because the pupal wing development was not disturbed in the by^2 mutants, we decided to investigate the expansion and unfolding processes of adult wings in the by^2 mutants. After eclosion, the by^2 flies displayed folded wings (Fig. 5F) similar to the control (Fig. 5A). Then, a sudden and rapid influx of hemolymph induced the unfolding of folded wings in the by^2 mutants (Fig. 5G) in the same manner as the control (Fig. 5B). However, as soon as the wings of by^2 flies unfolded, fluid-filled blisters began to appear at the distal part of the wings (Fig. 5H), and the boundary of the blisters expanded to a certain extent (Fig. 5I). After the fluid dried, the wing blisters were fixed in place (Fig. 5J).

Taken together, although the dorsal and ventral layers of a wing can be brought into close association during apposition and re-apposition processes in the by^2 flies, the link between them may not be strong enough to resist the hydrostatic pressures during the wing unfolding process.

The N-terminal region and the SH2 domain of tensin play a role in the attachment of the two wing surfaces

We examined the functional significance of each domain of tensin in normal wing development. We have generated UAS lines overexpressing either full-length tensin protein or various deletion mutant forms of tensin as shown in Fig. 6A. The ectopic expression of each transgene in the wing imaginal discs by MS1096-GAL4 driver was confirmed by RT-PCR (Fig. 6B). Unlike ΔN (Fig. 6D) and ΔC (Fig. 6E), overexpression of ΔPTB by MS1096-GAL4 driver completely rescued the blistered wing phenotype of by^2 (Fig. 6C). These data suggest that both the N-terminal region and the SH2 domain of tensin are required for proper attachment of two wing surfaces.

Tensin genetically interacts with integrin

As mammalian tensin is known to participate in the integrin signaling (Zamir and Geiger, 2001), we have examined whether tensin genetically interacts with integrin. As expected, the blistered wing phenotype became more severe in the if^3; by^2/+ mutants (Fig. 7C) and extremely severe in the double homozygotic mutants for if^3 and by^2 (Fig. 7D), compared with if^3 homozygotes (Fig. 7A) or by^2 heterozygotes (Fig. 7B). In addition, the rate of flies showing blistered wings in the total population greatly increased in the double mutants (Table 1).

To confirm that this enhanced severity of the phenotype in the if^3 by^2 double mutants was not due to an additive effect of two unrelated mutations, we tested the effect of heterozygous by^2 mutation on other wing blister-exhibiting mutants. Overexpression of rl^{Sem} using MS1096-GAL4 driver induced extra wing veins and sometimes resulted in a wing blister phenotype in about 21% of the total flies (Fig. 7E, Table 1). However, unlike the integrin mutants, the transgenic flies overexpressing rl^{Sem} within a heterozygotic by^2 genetic background did not significantly increase either the severity or the incidence of the blistered wing phenotype of flies

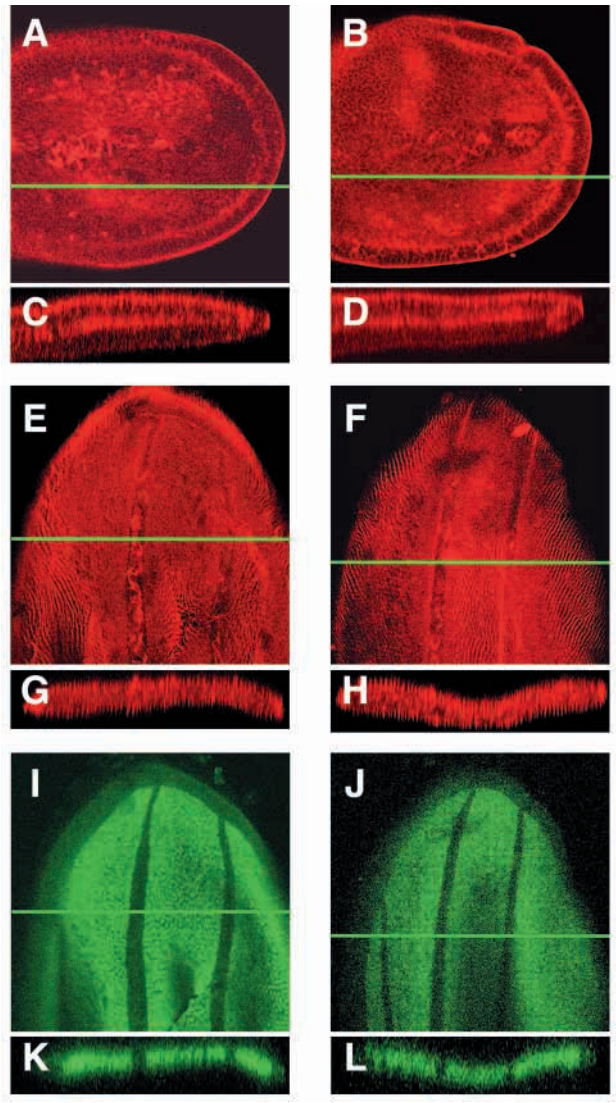

Fig. 4. Histochemical analyses of wing imaginal discs of by^2 flies. (A-D) Prepupal wings 4-6 hours after puparium formation. Wild-type (A) and the by^2 mutant (B) wings were stained with phalloidin-TRITC as described in the Materials and Methods. The same samples were optically cross-sectioned (C,D) in the regions indicated by the green horizontal lines (C,D). (E-L) Pupal wings at 30-36 hours after puparium formation. Wild-type (E,I) and the by^2 mutant (F,J) wings were stained for actin (E,F) and PS integrin (I,J) as described in the Materials and Methods. The same samples were optically sectioned. The corresponding optical sections for E,F,I,J are presented as G,H,K,L, respectively.

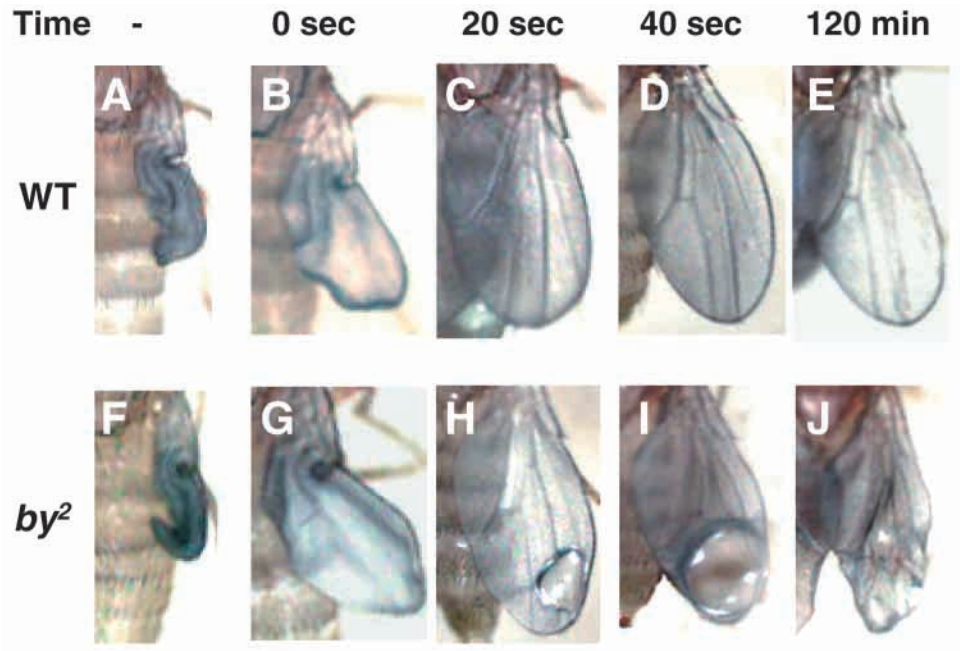

Fig. 5. Time-lapse pictures of the wing blister formation in the *by*^{2} mutants. Comparison of wild type (A-E) and the *by*^{2} mutants (F-J) at the same time scale. Pictures were taken immediately after eclosion (–) and at the indicated times after the initiation of wing unfolding.

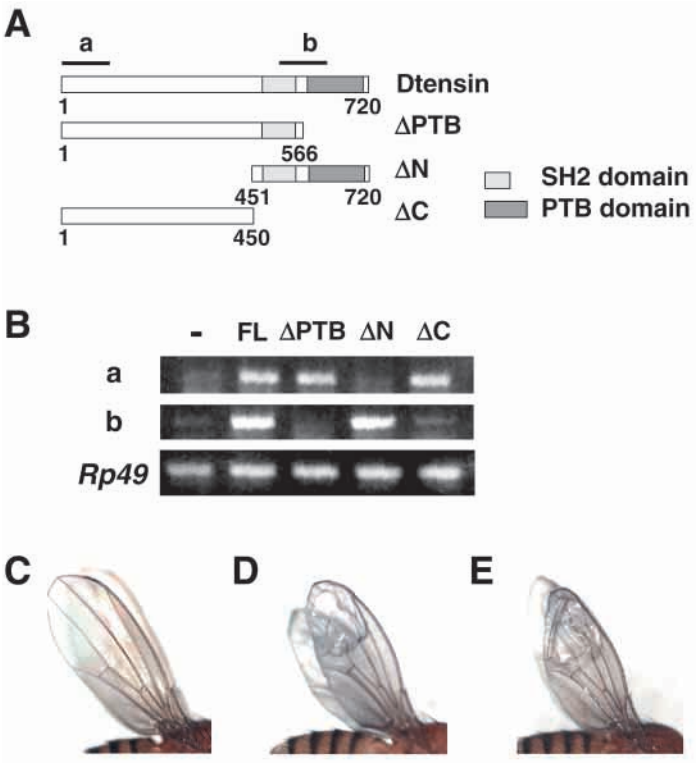

Fig. 6. The N-terminal region and the SH2 domain of tensin are required for normal wing development. (A) Various regions of *by* subcloned into pUAST vector were overexpressed in *Drosophila* as described in the Materials and Methods. (B) Determination of expression levels of *by* fragments in overexpression lines using RT-PCR method. 'a' and 'b' represent 5′ and 3′ regions of the *by* gene, respectively (shown in A). Ribosomal protein 49 (*Rp49*) was used as an internal control. FL, overexpression of full-length *by*. All RT products were amplified 25 cycles in PCR reactions. (C) MS1096/X; UAS-ΔPTB/+; *by*^{2}/ *by*^{2}. (D) MS1096/X; *by*^{2},UAS-ΔN/*by*^{2}. (E) MS1096/X; UAS-ΔC/+; *by*^{2}/*by*^{2}.

overexpressing *rl*^{Sem} (Fig. 7F, Table 1). Collectively, these results suggest that tensin functionally interacts with integrin in the wing development of *Drosophila* in a specific manner.

Tensin genetically interacts with the JNK signaling pathway

In mammalian cells, tensin has been implicated in signal transduction related to cell adhesion such as Src, JNK and PI3K (Thomas et al., 1995; Auger et al., 1996; Katz et al., 2000). To examine the role of tensin in the signaling processes related to wing development, we investigated the in vivo interaction between tensin and signaling molecules including rl/Erk, Src, JNK and PI3K. Interestingly, we found that the JNK signaling pathway is tightly correlated with tensin in the wing development (Fig. 8), while other signaling molecules including rl/Erk did not show any interactions with tensin (Fig. 8I,J, and data not shown). Homozygous *by*^{2} mutants with heterozygotic mutations of the JNK signaling components *bsk*^{1} (Fig. 8B, Table 1) or *hep*^{1} (Fig. 8E,F, Table 1) (the loss-of-function mutants for *Drosophila JNK* and *MKK7*, respectively) displayed a highly severe blistered wing phenotype compared with either homozygous *by*^{2} (Fig. 2A, Table 1), heterozygous *bsk*^{1} (Fig. 8A, Table 1) or heterozygous *hep*^{1} (Fig. 8D, Table 1) mutants. Notably, the rate of flies, which showed Class II blistered wings, increased from 46.5% to 70% for these double mutants compared with homozygous *by*^{2} mutants, and about 15% of these flies had multiple blisters in their wings. Furthermore, the double homozygotic mutants for *by*^{2} and *hep*^{1} died at pharate adult stage (Fig. 8C). The lethality of these double mutants may be due to an impairment of essential in vivo interactions between tensin and the JNK signaling pathway in *Drosophila*.

Next, we tested whether overexpressed *by* also interacts with the components of the JNK signaling pathway. Overexpression of *by* using MS1096-GAL4 driver turned the adult wings into a convex shape with a smaller overall size (Fig. 8G), and this

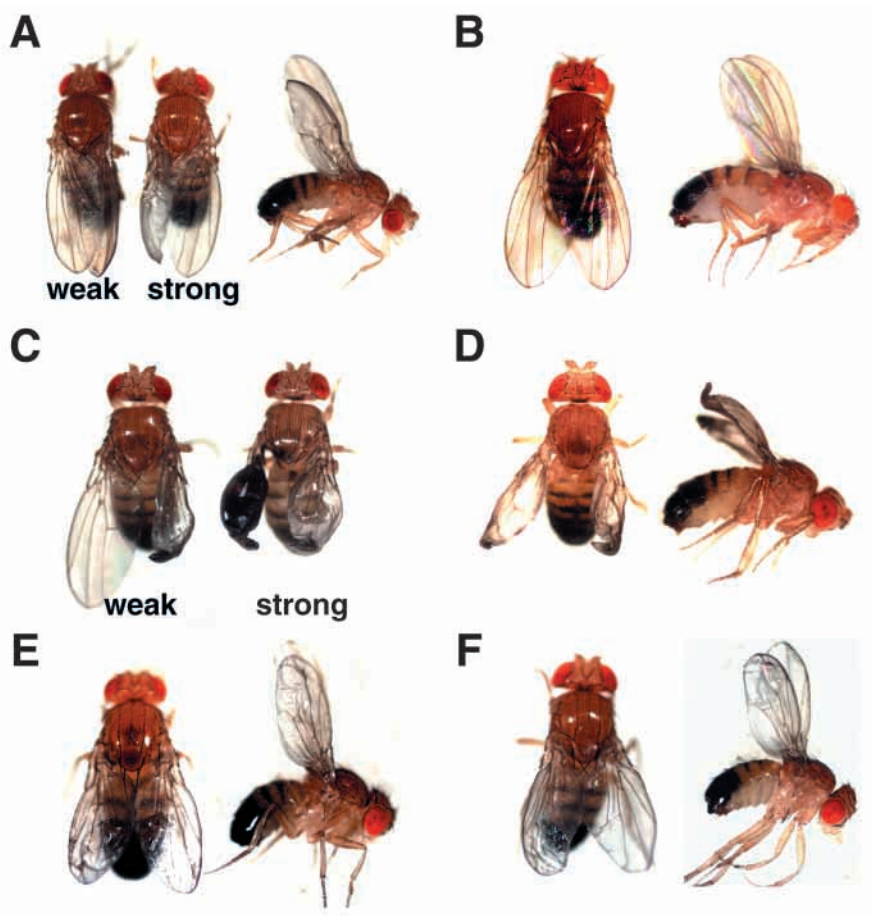

Fig. 7. Genetic interactions between *Drosophila* integrin and tensin. (A) if^3/Y. (B) by^2/+. (C) if^3/Y; by^2/+. (D) if^3/Y; by^2/by^2. (E) MS1096/Y; UAS-rl^{Sem} /+. (F) MS1096/Y; UAS-rl^{Sem} /by^2.

phenotype became more severe when two copies of the *by* gene were overexpressed (Fig. 8H). Simultaneous overexpression of *bsk* (Fig. 8L) or *hep* (Fig. 8N) with *by* resulted in a severely curled wing phenotype, which was fully penetrant, whereas overexpression of *bsk* (Fig. 8K) or *hep* (Fig. 8M) alone by MS1096-GAL4 driver did not induce any detectable phenotypes in the wing. Collectively, these data suggest that tensin activity is highly related to the JNK signaling pathway during wing development in *Drosophila*.

Table 1. The rates of flies showing blistered wings

Genotype	Blistered (%)*	$n^{\dagger}$
if^3/Y	28.0	400
X/Y; by^2/+	0	784
if^3/Y; by^2/+	70.0	784
X/Y; by^2/by^2	95.3	392
if^3/Y; by^2/by^2	100	392
MS1096; UAS-rl^{Sem}/+	20.9	210
MS1096; UAS-rl^{Sem}/by^2	24.6	210
bsk^1/+; +/+	0	120
hep^1/Basc; +/+	0	120
bsk^1/+; by^2/by^2	100	120
hep^1/Basc; by^2/by^2	100	120

*(The number of flies with blistered wings/n) × 100.
†Total number of observed flies of indicated genotype.

Overexpression of *by* induces the activation of JNK and ectopic apoptosis

To further confirm the genetic interaction between tensin and the JNK pathway, we measured the effect of tensin in JNK activity in vivo. We examined the extent of JNK phosphorylation using anti-phosphospecific JNK antibody in the *by* overexpression line and the by^2 mutants. As expected, JNK phosphorylation was dramatically increased in the wing imaginal discs overexpressing *by* (Fig. 9C) compared with the control (Fig. 9A), which directly demonstrated increased JNK activity by *by*. On the contrary, JNK phosphorylation in the imaginal discs of the by^2 mutants was reduced (Fig. 9B) compared with the control (Fig. 9A).

Moreover, the reduced size and the convex wing phenotype observed in the wings overexpressing *by* (compare Fig. 9E with its control Fig. 9D) can be most easily explained by apoptosis in the wings. As the induction of apoptosis by the JNK signaling is well established (Goberdhan and Wilson, 1998; Adachi-Yamada et al., 1999), we expected that the wing phenotype induced by *by* overexpression might be due to apoptosis. To confirm the *by*-induced apoptosis in vivo, we carried out Acridine Orange staining of the relevant wing imaginal discs. As expected, the overexpression of *by* dramatically increased apoptotic cell death (Fig. 9G) compared with the control (Fig. 9F).

DISCUSSION

In this study, we have investigated the function of *Drosophila* tensin ortholog (*by*) by analyzing its loss-of-function and gain-of-function mutants. By observing the role of tensin in the development of the fly wing and by analyzing its genetic interactions with various signaling components, we were able to gain valuable insights into the in vivo roles of tensin.

The N-terminal region of tensin is responsible for the wing blister phenotype of the *by* mutants

Our current data demonstrate that by^1 allele contains two missense mutations in the N-terminal region of tensin, and that the expression of *by* is not affected in the by^1 mutants compared with the wild-type control (Fig. 3C). Because the severity of the wing blister phenotype in by^1 was not significantly different from the *by*-deficient mutants such as by^2 and by^{ex49} (Fig. 3B), and the ectopic expression of ΔN form of tensin did not rescue the blistered wing phenotype of by^2 (Fig. 6), we think that the mutations in the N-terminal region of *Drosophila* tensin including tyrosine 62 and threonine 163 are responsible for the wing blister phenotype.

However, as Tyr62 and Thr163 are not conserved in other tensins, and there is no information for unmutagenized parental chromosomes of by^1, further studies are required to verify the

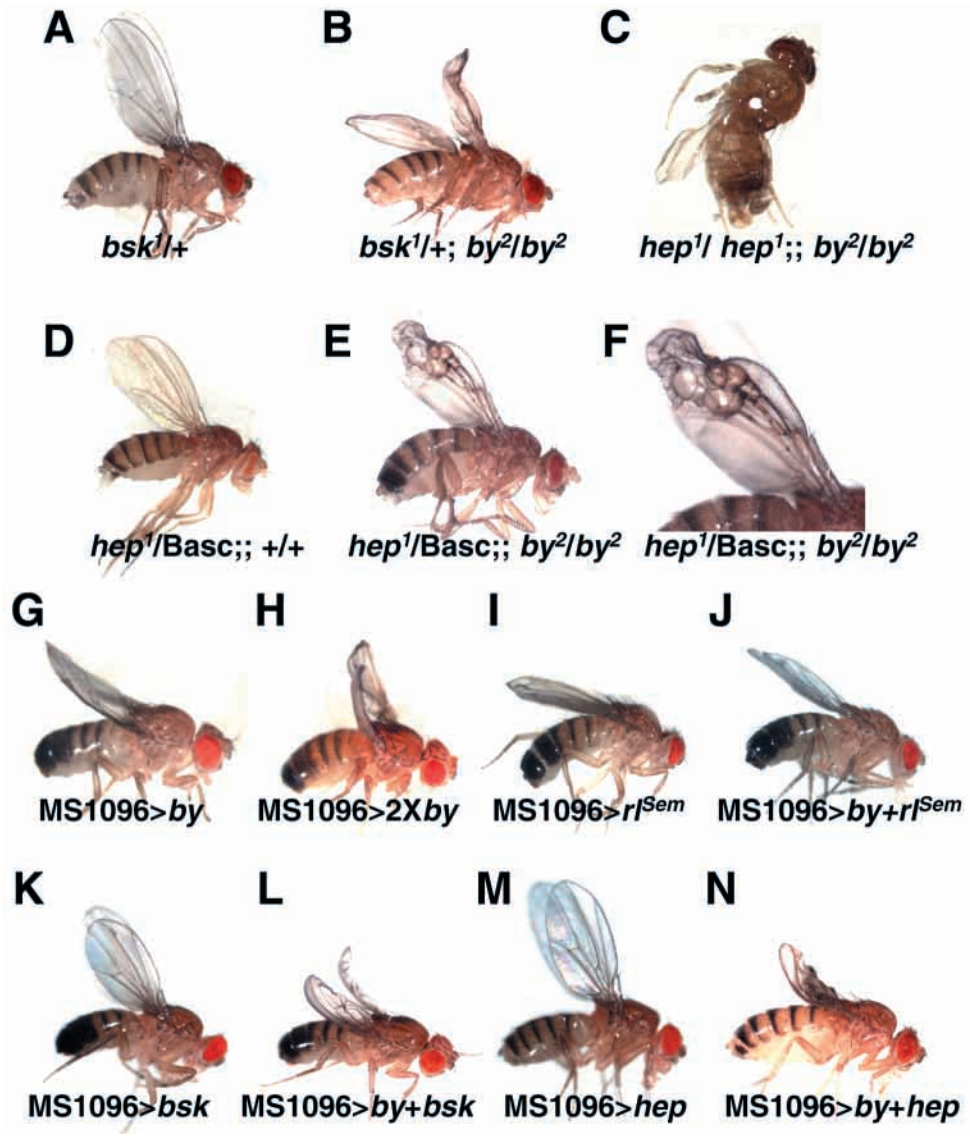

Fig. 8. Functional interactions between tensin and the JNK pathway. (A) *bsk*^{1}/+. (B) *bsk*^{1}/+; *by*^{2}/*by*^{2}. (C) *hep*^{1}/ *hep*^{1}; *by*^{2}/*by*^{2} (pharate pupa). (D) *hep*^{1}/Basc; +/+. (E,F) *hep*^{1}/Basc; *by*^{2}/*by*^{2}. (G) MS1096/Y; UAS-*by*/+. (H) MS1096/Y; UAS-*by*/UAS-*by*. (I) MS1096/Y; UAS-*rl*^{Sem}/+. (J) MS1096/Y; UAS-*rl*^{Sem}/UAS-*by*. (K) MS1096/Y; UAS-*bsk*/+. (L) MS1096/Y; UAS-*bsk*/+; UAS-*by*/+. (M) MS1096/X; UAS-*hep*/+. (N) MS1096/X; UAS-*hep*/+; UAS-*by*/+.

exact nature of *by*^{1} mutations, and to address the molecular mechanism underlying how mutations of *by*^{1} allele affect the cellular functions of tensin.

Tensin cooperates with integrin for the proper attachment of wing epithelia

During *Drosophila* wing development, the dorsal and ventral wing epithelia are fused together by highly specified cell-cell adhesions, and defects in this process result in blistered wings. Therefore, the blistered wing phenotype observed in the *by* mutants indicates that tensin functions in such a cell adhesion process.

In *Drosophila*, integrin is well known as a central molecule that mediates adhesion between wing layers. Previous studies have shown that the loss of PS integrin function in the wings causes the formation of a fluid-filled blister (Brower and Jaffe, 1989; Wilcox et al., 1989; Brabant and Brower, 1993; Brown et al., 2000). Moreover, the loss of adaptor proteins such as *short stop* (Prout et al., 1997) and *integrin-linked kinase* (Zervas et al., 2001) that mediate attachment of extracellular matrix (ECM)-integrin complexes to cytoskeleton also result in a wing blister phenotype, implying that integrin and its adaptor protein complexes are indispensable in wing layer adhesion. Because tensin is a major component of the ECM-integrin complex, and the wing blister phenotype of an integrin mutant was dramatically enhanced by additional loss of *by* in this study (Fig. 7 and Table 1) we believe that improperly mediated integrin signaling caused by the loss of tensin results in the wing blister phenotype in the *by* mutants. This idea is supported by previous mammalian cell studies showing that tensin acts as a molecular linker between actin cytoskeleton and integrin (Zamir and Geiger, 2001), and plays a crucial function when integrin translocates from focal adhesions to fibrillar adhesions (Pankov et al., 2000).

Besides the defects in wing cell adhesion process, we observed another distinct mutant phenotype in the *by* mutants; they laid rounded eggs due to defective oocyte elongation during oogenesis (K.S.C., unpublished). Our unpublished genetics data suggest the possibility of a functional interaction between tensin and integrin during oogenesis. Interestingly, a similar phenotype has been reported recently in the studies with follicle cell clones lacking PS integrin (Bateman et al., 2001).

Taken together, above findings suggest that tensin and integrins are tightly linked together during most, if not all, of their various functions in *Drosophila* development.

Tensin regulates JNK activity during wing development

In this study, tensin was observed to genetically interact with the components of the JNK signaling pathway, and regulate JNK activity during wing development (Figs 8, 9). The supporting evidence for the engagement of tensin in the JNK signaling pathway comes from a recent report that transfected mammalian tensin activates JNK signaling in HEK 293T cells (Katz et al., 2000).

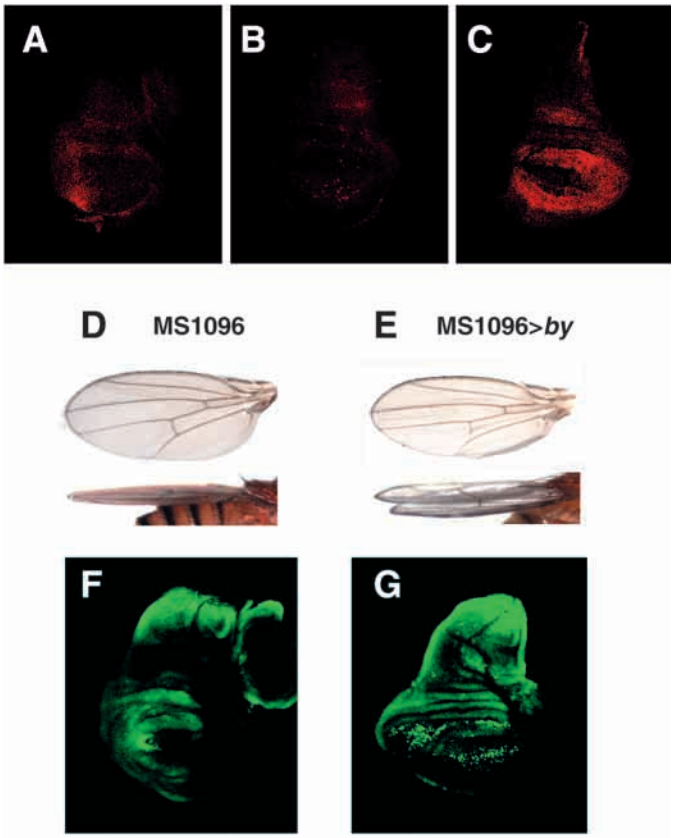

Fig. 9. Induction of apoptosis and JNK activity by *by*. (A-C) In immunohistochemical analyses using anti-phosphospecific JNK antibody, JNK phosphorylation was dramatically increased in the wing imaginal discs expressing *by* (C), and decreased in the wing imaginal discs of homozygous by^2 mutant (B) compared with the wild-type control (A). (D) The MS1096-GAL4 fly displayed a normal wing phenotype. (E) Overexpression of *by* using MS1096-GAL4 turned the wing into a convex shape. (F,G) In Acridine Orange staining experiments, overexpression of *by* (G) dramatically induced apoptotic cell death in wing imaginal discs, compared with the wild-type control (F).

Interestingly, in mammalian cells, JNK is also activated via adaptor proteins p130 CAS and Crk which receive a signal from the FAK/Src tyrosine kinase complex in the cell adhesion sites when cells attach to the ECM (Oktay et al., 1999). As tensin is a possible substrate for FAK (Guan, 1997), and p130 CAS is able to interact with the C terminus of tensin (Salgia et al., 1995; Salgia et al., 1996), it is highly possible that tensin is involved in this signaling cascade and mediates signals from integrin and FAK to the JNK signaling pathway.

In addition, tensin was observed not to genetically interact with other signaling pathways known to interact with integrins such as the ERK-MAPK (Fig. 8) and the PI3K signaling pathways (data not shown). As previously mentioned, integrin signaling is mediated mainly by protein complexes including tensin in focal adhesions. Thus, we think that focal adhesion molecules related to integrin such as tensin are important for directing integrin mediated extracellular signals to a specific signaling pathway. Consequently, we tentatively suggest that at least during *Drosophila* wing development, tensin has an ability to drive signals from integrin selectively to the JNK signaling pathway. However, further studies are required to confirm this hypothesis and determine the details behind the connection between focal adhesion proteins and related intracellular signaling.

In summary, we have characterized *by* mutant flies and analyzed the developmental roles of *by* specifically in wings. We found evidence for the functional interaction between integrin and tensin, and for the modulation of JNK signaling by tensin during *Drosophila* wing development. These results strongly suggest that tensin is not merely an adaptor protein in focal adhesions, but also an important mediator of signal transduction in *Drosophila*. Our *Drosophila* model will be useful in future studies that address the function of tensin as a signaling molecule.

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