Modulation of Integrin Activity is Vital for Morphogenesis

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Abstract. Cells can vary their adhesive properties by modulating the affinity of integrin receptors. The activation and inactivation of integrins by inside-out mechanisms acting on the cytoplasmic domains of the integrin subunits has been demonstrated in platelets, lymphocytes, and keratinocytes. We show that in the embryo, normal morphogenesis requires the α subunit cytoplasmic domain to control integrin adhesion at the right times and places. PS2 integrin ($\alpha_{PS2}\beta_{PS}$) adhesion is normally restricted to the muscle termini, where it is required for attaching the muscles to the ends of other muscles and to specialized epidermal cells. Replacing the wild-type α_{PS2} with mutant forms containing cytoplasmic domain deletions results in the rescue of the majority of defects associated with the absence of the α_{PS2} subunit, however, the mutant PS2 integrins are excessively active. Muscles containing these mutant integrins make extra muscle attachments at aberrant positions on the muscle surface, disrupting the muscle pattern and causing embryonic lethality. A gain-of-function phenotype is not observed in the visceral mesoderm, showing that regulation of integrin activity is tissue-specific. These results suggest that the α_{PS2} subunit cytoplasmic domain is required for inside-out regulation of integrin affinity, as has been seen with the integrin $\alpha_{IIb}\beta_3$.

CHANGES in cell adhesiveness in response to developmental cues are critical in cell migration and differentiation. Cell adhesion is controlled by modulating the binding properties of cell surface receptors and their ligands. One of the first instances of this regulation occurring in embryogenesis happens during compaction of the early mouse embryo to form the blastocyst. The surface expression of the cell adhesion molecule E-cadherin does not change, but instead is activated at the eight-cell stage by intracellular events (Fleming and Johnson, 1988). Another family of cell adhesion receptors that undergoes modulation of activity is the integrins, which play a major role in cell–cell contact and in interactions between cells and the extracellular matrix (Hynes, 1992). In a number of cases it has been shown that integrin affinity for extracellular ligands is modulated by an intracellular mechanism (inside-out signaling), and that this modulation is correlated with changes in cellular behavior. During blood clotting, platelet binding to fibrinogen is achieved by activating the $\alpha_{IIb}\beta_3$ integrin, which is already present on the cell surface in an inactive form (Manning and Brass, 1991; Shattil and Brugge, 1991). The β_2 integrins expressed on leukocytes undergo activation in a similar way to that occurring in $\alpha_{IIb}\beta_3$ integrins (Arnaout, 1990; Larson and Springer, 1990). At sites of inflammation, the β_2 integrins, which are in an inactive state on circulating leukocytes, are exposed to and activated by inflammatory mediators during rolling, a low-affinity adhesion step mediated by selectins. An analogous situation exists for T lymphocytes, where cross-linking of CD3 or the costimulating receptor CD2 leads to activation of $\alpha_L\beta_2$ integrin on the T cells (van Kooyk et al., 1989). In both cases, activation of integrins is mediated by an adhesion and intracellular signaling cascade that involves protein kinase C (Butcher, 1991; Dustin and Springer, 1991). Modulation of β_1 integrin activity has also been found to occur during the final stage of terminal differentiation in keratinocytes. In this case, keratinocyte detachment from the basement membrane correlates with loss in the ability of the β_1 integrins to bind ligands, reduced transport of newly synthesized subunits to the cell surface, and loss of mature integrins from the cell surface (Adams and Watt, 1990; Hotchin and Watt, 1992; Hotchin et al., 1995).

It seems vital to ensure that integrins are only activated at the right times and places. For example, inappropriate activation of $\alpha_{IIb}\beta_3$ integrin on resting circulatory platelets may lead to thrombosis (Kieffer and Phillips, 1990; Kieffer et al., 1991), and activation of the β_2 integrins at the wrong places on leukocytes may lead to inflammation (Arnaout, 1990; Larson and Springer, 1990). To confirm this hypothesis, it is important to test these models in vivo.

Integrins cytoplasmic domains are likely targets of cytoplasmic signals that alter integrin affinity (reviewed in Sas-

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try and Horwitz, 1993). Requirements for β intracellular domain function have been demonstrated for cell adhesion (Hayashi et al., 1990; Hibbs et al., 1991), cell spreading (Ylanne et al., 1993), and adhesion-dependent phosphorylation of pp125 focal adhesion kinase (Guan et al., 1991; Hanks et al., 1992). A variety of experiments in cell culture have shown that the α cytoplasmic domains of integrins also play critical roles in determining adhesive activity. However, the results are somehow contradictory. It has been shown that deletion of the α_2, α_4, or α_6 tails after the highly conserved α subunit membrane proximal motif GFFKR leads to a loss of adhesive activity (Kassner and Hemler, 1993; Kawaguchi and Hemler, 1993; Shaw and Mercurio, 1993; Kassner et al., 1994). Conversely, deletion of the α_L, α_1, or α_5 cytoplasmic domains does not affect the ability of the mutant $\alpha_L\beta_2$, $\alpha_1\beta_1$, or $\alpha_5\beta_1$ receptors to mediate adhesion to their respective ligands (Hibbs et al., 1991; Bauer et al., 1993; Briesewitz et al., 1993). In addition, in the only system where mutations in integrin subunits have been tested for their effect on inside-out signaling, truncating the α_{IIb} cytoplasmic domain mimics inside-out activation and results in an integrin that constitutively binds its ligand fibrinogen (O'Toole et al., 1991). A mutation that just deletes the α cytoplasmic GFFKR motif also results in a constitutively high-affinity $\alpha_{IIb}\beta_3$ integrin (O'Toole et al., 1994). Therefore, inside-out signaling appears to occur by modulating the α cytoplasmic tail, since deleting it mimicks inside-out activation. We were interested in determining whether the cytoplasmic domains of integrin α subunits are required for regulation of integrin activity during embryogenesis. In *Xenopus*, changes in cellular adhesion to fibronectin are correlated with increased migration of cells during gastrulation, suggesting that inside-out integrin modulation may play an important role in this developmental event (Ramos et al., 1996). We wished to test whether the ability of integrins to be modulated by intracellular interactions with the α subunit is not only correlated with morphogenetic events, but is essential for them to occur.

Identification and characterization of the *Drosophila* position-specific (PS)[1] integrins provides the opportunity to examine integrin function in the developing organism. The two PS integrins, PS1 and PS2, share a common β subunit (β_{PS}), but have different α subunits (α_{PS1} and α_{PS2}). The *Drosophila* PS integrins have amino acid sequence homology with vertebrate integrins, and structural and functional features of the vertebrate integrins are conserved in the fly proteins (Bogaert et al., 1987; MacKrell et al., 1988; Hirano et al., 1991; Bunch and Brower, 1992; Wehrli et al., 1993). Throughout development, the two PS integrins are expressed in complementary but closely associated tissues. For example, high levels of PS integrins are found at sites where the muscles attach to the epidermis, with $\alpha_{PS1}\beta_{PS}$ (PS1) restricted to the epidermal cells, and $\alpha_{PS2}\beta_{PS}$ (PS2) to the muscle cells (Bogaert et al., 1987; Leptin et al., 1989; Wehrli et al., 1993). An analogous situation occurs in the midgut, with PS1 expressed in the gut endothelium and PS2 in the visceral mesoderm that surrounds the gut (Bogaert et al., 1987; Leptin et al., 1989). Loss of function mutations in *inflated* (*if*; α_{PS2} subunit gene) have demonstrated that the PS2 integrin is required in formation and maintenance of somatic muscle attachments to the epidermis, and the attachment of the visceral muscles to the midgut epithelium (Babrant and Brower, 1993; Brown, 1994; Prokop et al., 1998).

Although the roles of PS integrins during embryonic development have been intensively studied, we do not know if their function is regulated during development. It has been shown that the cytoplasmic domain of the β_{PS} integrin subunit is essential during development for β_{PS} functions (Grinblat et al., 1994). Futhermore, we have shown that this domain is sufficient to localize a chimeric protein to the end of the muscles, demonstrating the existence of an inside-out mechanism able to localize the PS2 integrin (Martin-Bermudo and Brown, 1996). In this work we test whether this or other inside-out signaling processes are used to modulate PS2 integrin activity. Since a deletion of the α cytoplasmic domain has generated a constitutively active $\alpha_{IIb}\beta_3$ integrin (O'Toole et al., 1994), we tested whether a similar mutation in the α subunit of the PS2 integrin would cause developmental defects, indicating that modulation of integrin activity is essential for embryonic development. We show that deletion of the α_{PS2} cytoplasmic domain leads to formation of a PS2 integrin that mediates formation of an abnormal number and size of muscle attachments consistent with PS2 being constitutively activated. This result demonstrates that modulation of integrin function through the cytoplasmic domain of the α subunit is essential for embryonic morphogenesis.

Materials and Methods

Preparation of Mutant Integrins

The UAS-α_{PS2} cytoplasmic tail mutants were generated by site-directed mutagenesis using the technique described by Picard et al. (1994). The two mutations were made by: (a) inserting a stop codon at the end of exon 11 so a truncated form of the protein would be produced (Δcyt); and (b) deleting the highly conserved GFFNR motif (ΔGFFNR). To make the Δcyt mutant, we amplified a portion of the α_{PS2} gene from a subclone of α_{PS2} encoding the COOH end (part of exon 11 and 12) using the PCR. For this amplification we used three primers: two flanking primers (vector sequence) that were upstream (P1) and downstream (P2) of the mutation site, and a mutant primer that introduced a stop codon at the end of exon 11 (5′GG CTG CTC TAC AAG TAG GAT CCT TAA CCC TTT CTC TCG G 3′). The mutant primer and P2 were used to produce a mega primer, which was then used in conjunction with P1 to produce the desired fragment. This PCR fragment was digested with BglII and SalI, gel-purified, subcloned, and checked by sequencing. This fragment was then used to replace the corresponding fragment in the wild-type UAS-$\alpha_{PS2c/g}$ construct (Martin-Bermudo et al., 1997). To obtain the ΔGFFNR construct, the same procedure was followed as for Δcyt, but in this case the mutant primer was missing the bases encoding the GFFNR motif at the start of exon 12 (5′ CCT TTA ACC CTA CAG TGC AAC CGG CCA ACG GAT CAC TCG C 3′). To generate germline transformants, both constructs were injected into flies using standard methods.

The construction of the minigene has been described (Bloor and Brown, 1998). To generate the ΔGFFNR mutant form of this minigene, we replaced a SacII-RsrII fragment in the wild-type minigene with that from the UAS-$\alpha_{PS2\Delta GFFNR}$.

Drosophila Strains

The integrin mutant allele used in this study is the null allele if^{B4} (Brown, 1994) marked with *y w f*. Since *if* is on the X chromosome, to select the mutant embryos we have used two derivatives of the *FM6* balancer—a y^+ derivative and a *lacZ* marked derivative—and they have been used as de-

1. *Abbreviation used in this manuscript*: PS, position-specific.

scribed in Martin-Bermudo et al., (1997). To assay rescue of *inflated* embryonic lethality, a 4-h collection of embryos at 28°C was transferred to new apple juice plates in groups of 20 aged for 24 h, and the embryos that failed to hatch were counted. In these experiments, we distinguished the mutant embryos with the *y* marker. We have used the following independent inserts for the different constructs: UAS-$\alpha_{PS2c/g}$: 3.A (Martin-Bermudo et al., 1997); UAS-$\alpha_{PS2\Delta cyt}$: 1, 2, 6; UAS-$\alpha_{PS2\Delta GFFNR}$: 4, 6; minigene-α_{PS2}: 47, 55; and minigene-$\alpha_{PS2\Delta GFFNR}$: 76, 96.

Histology

Whole mount staining of embryos was performed using standard procedures. The primary antibodies used were the CF6G11 mouse mAb against β_{PS} (1:1,000; Brower et al., 1984), the 7A10 PS2 hc/2 rat mAb against α_{PS2} (1:5; Bogaert, et al., 1987), anti-muscle myosin (Kiehart and Feghali, 1986), a mouse anti-gp150 (Fashena and Zinn, 1997), and anti-β-galactosidase (Cappel Laboratories, Malvern, PA). We used a biotin-labeled secondary antibody followed by the Vectastain Elite ABC Kit (Vector Labs, Inc., Burlingame, CA) enhancement to stain the embryos. To visualize the visceral mesoderm, dissected guts were fixed in 5% formaldehyde in PBT for 20 min and stained with rhodamine-labeled phalloidin as described in Xue and Cooley (1993). Images were obtained by photography on a Zeiss Axiophot followed by scanning with a Nikon Coolscan (Nikon Inc., Instrument Group, Melville, NY), or directly from the MRC1000 Confocal™ microscope (Bio-Rad Laboratories, Hercules, CA). Images were assembled in Photoshop 3.0 (Adobe Systems, Inc., Mountain View, CA) and labeled in Freehand 5.5™ (Macromedia, San Francisco, CA).

Results

Expression of a PS2 Integrin with Deletions Within the α Cytoplasmic Domain in the Developing Embryo

Two mutant α_{PS2} subunit genes containing deletions within the cytoplasmic domain were constructed (Fig. 1). In one construct, we have deleted the entire cytoplasmic domain ($\alpha_{PS2\Delta cyt}$), and in the other we removed the α_{PS2} variant form of the highly conserved domain GFFKR ($\alpha_{PS2\Delta GFFNR;}$ see Materials and Methods). We have already successfully used the GAL4 system (Brand and Perrimon, 1993) to rescue the embryonic lethality of *inflated* mutant embryos that lack the α_{PS2} subunit by expressing a wild-type α_{PS2} construct in the mesoderm using a combination of two GAL4 lines: *twist-GAL4* and *24B* (Martin-Bermudo et al., 1997). We have previously shown that in *inflated* mutant embryos, the β_{PS} subunit is not found at the end of muscles, but remains within the endoplasmic reticulum (Martin-Bermudo et al., 1997; and Fig. 2 *b*). We have also shown that we can restore wild-type levels of β_{PS} localization to the ends of muscles in mutant embryos by expressing the α_{PS2} subunit with the GAL4 system. To analyze the role of the cytoplasmic domain of the α_{PS2} subunit in regulating PS2 integrin function during embryogenesis, we have used the same GAL4 lines—*twist-GAL4* and *24B*—to express the truncated forms of the α_{PS2} subunit in the mesoderm of *inflated* mutant embryos. To examine whether the mutant α_{PS2} subunits are able to form heterodimers with the β_{PS} that are properly localized, we stained the different transgenic embryos with an anti-β_{PS} antibody. When we express either of the two truncated forms of the α subunit in these mutant embryos, we find that they can also restore β_{PS} localization (Fig. 2 *c* and data not shown). From these results we conclude that α_{PS2} subunits containing deletions in the cytoplasmic domain are able to form heterodimers with the β_{PS} subunit that are properly localized to the ends of the muscles. We then tested whether these mutant integrin heterodimers are able to function in the developing embryo.

Regulation of PS2 Integrin Activity Through the α Subunit Cytoplasmic Domain is Essential for Normal Morphogenesis

Experiments with cells in culture have shown that integrin α cytoplasmic domains can regulate the ligand-binding function of their extracellular domains (reviewed in Ginsberg, et al., 1992; Hynes, 1992); however, the results obtained vary depending on the integrin examined. As mentioned in the introduction, deletion of α cytoplasmic domains can have three different effects on the adhesive activity of the integrin: to alter it, abolish it, or constitutively active it. Therefore, it was not simple to predict the consequences that mutating the cytoplasmic domain of the α_{PS2} subunit would have on embryonic development.

Expression of the $\alpha_{PS2\Delta GFFNR}$ mutant subunit in embryos that lack α_{PS2} rescues the if^{B4} embryonic lethality almost as well as the wild-type construct (Fig. 3; Martin-Bermudo et al., 1997). However, if we express the $\alpha_{PS2\Delta cyt}$ mutant form, we find that it is unable to rescue the lethality caused by the absence of α_{PS2} (Fig. 3). This result is not due to lower levels of expression of this mutant integrin, since using two copies of the UAS-$\alpha_{PS2\Delta cyt}$ transgene does not significantly improve the ability of this mutant form to rescue if^{B4} (Fig. 3). The failure of the Δcyt truncated form of the α_{PS2} subunit to rescue the *inflated* embryonic lethality could be due to the fact that deletion of the cytoplasmic domain creates a nonfunctional α_{PS2} subunit. Alternatively, this deletion could create a constitutively active receptor, which causes the lethal phenotype. To distinguish between these two possibilities, we examined the muscle attachment phenotype of embryos in which the wild-type α_{PS2} subunit has been replaced with the truncated forms of the α_{PS2} subunit.

In a wild-type *Drosophila* embryo, mononucleated myoblasts fuse to form myotubes, and during this process they elongate and attach to specific sites on the basal surface of the epidermis (apodemes). Initially, one pole of the mus-

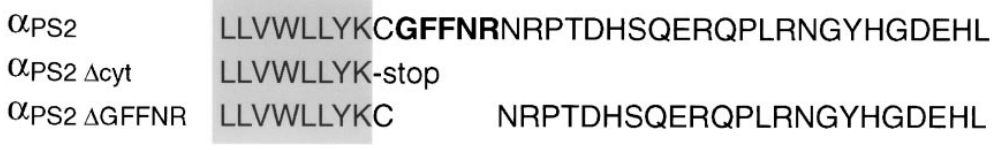


Figure 1. Amino acid sequence of the two mutated α_{PS2} cytoplasmic domains. The cytoplasmic tail and part of the transmembrane region of α_{PS2} subunit are shown at the top. Beneath this, the two mutants generated are shown: αPS2Δcyt and αPS2ΔGFFNR.

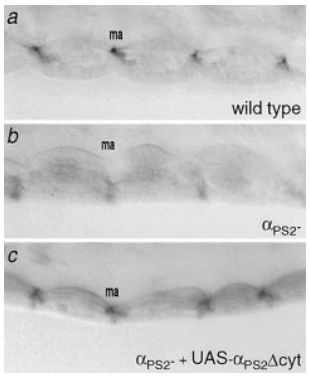


Figure 2. The α_{PS2} cytoplasmic domain is not required for localization of the PS2 integrin to the end of the muscles. An antibody against the β_{PS} subunit was used to visualize expression of the PS2 integrin. (*a*) In a wild-type embryo, the PS2 integrin is localized at the muscle attachment sites (*m.a.*). (*b*) In mutant embryos that lack the α_{PS2} subunit, the β_{PS} staining at the end of the muscles is lost. (*c*) Expression of the UAS-$\alpha_{PS2\Delta cyt}$ construct in an α_{PS2} mutant embryo produces a mutant heterodimer that is correctly localized at the muscle termini.

cle is attached to the epidermis at one apodeme, and the other consists of a growth cone–like structure that migrates towards the other apodeme (Bate, 1990). Additional membrane projections are also observed on the lateral surfaces of the muscles, but these decrease as the growth cone is established. By the end of stage 15, approximately half way through embryogenesis, most of the muscles have reached their mature size, their surfaces no longer show any membrane projections, and both ends are stably associated with their respective apodemes. In embryos mutant for the PS2 integrin, the muscles then begin to detach from the apodemes, showing that the PS2 integrin is essential for formation of strong muscle attachments, but not for their initial formation (Wright, 1960; Brown, 1994).

To look at the ability of the truncated forms of the α_{PS2} subunit to rescue this muscle detachment phenotype, we have stained *if* mutant embryos carrying the different transgenes with an anti-muscle myosin antibody (Fig. 4). In the absence of any UAS construct, the *if* mutant embryos show a muscle detachment phenotype, as expected (Fig. 4 *b*). The expression of a wild-type UAS-α_{PS2} construct completely rescues the muscle phenotype (Martin-Bermudo et al., 1997). *if* mutant embryos carrying the UAS-$\alpha_{PS2\Delta GFFNR}$ construct are almost completely rescued, but they have a very mild muscle detachment phenotype, with just one muscle detached in 15% of the mutant embryos (not shown). In contrast, we could not detect any muscle detachment phenotype in those *if* mutant embryos carrying the UAS-$\alpha_{PS2\Delta cyt}$ construct (Fig. 4 *c*), despite the failure of this construct to rescue embryonic lethality.

Closer examination revealed that both mutant forms of the α_{PS2} subunit cause two new phenotypes. First, the muscles make attachments that are larger than in wild-type embryos, as can be seen particularly clearly for the transverse muscles, which have broader tips (compare enlargements in Fig. 4, *a* and *c*). Second, the muscles of these embryos form abnormal attachments along the lateral surfaces. For example, the wild-type ventral longitudinal muscles (VL) normally extend the whole length of each segment and attach to apodemes at the segment boundary, while ventral longitudinal muscles containing the mutant PS2 integrins make new attachments to the ventral acute muscles (VA) in the middle of the segment (*arrowheads* in Fig. 4, *d* and *e*). Another aspect of this phenotype is the aberrant processes that are found extending from the lateral surfaces of the VL towards the lateral transverse muscles (LT), as seen with an antibody against the transmembrane glycoprotein gp150 (Fashena and Zinn, 1997) that stains the surface of muscles (Fig. 5). The mutant $\alpha_{PS2\Delta cyt}\beta_{PS}$ integrin is found at the ectopic attachment sites (Fig. 4 *d*), and the extracellular matrix protein Tiggrin (Fogerty et al., 1994), a PS2 ligand, is also recruited (Fig. 4 *e*). Although staining of both PS2 and Tiggrin at the ectopic site appears modest, it is the amount expected for the end of a single muscle, compared with the segment border

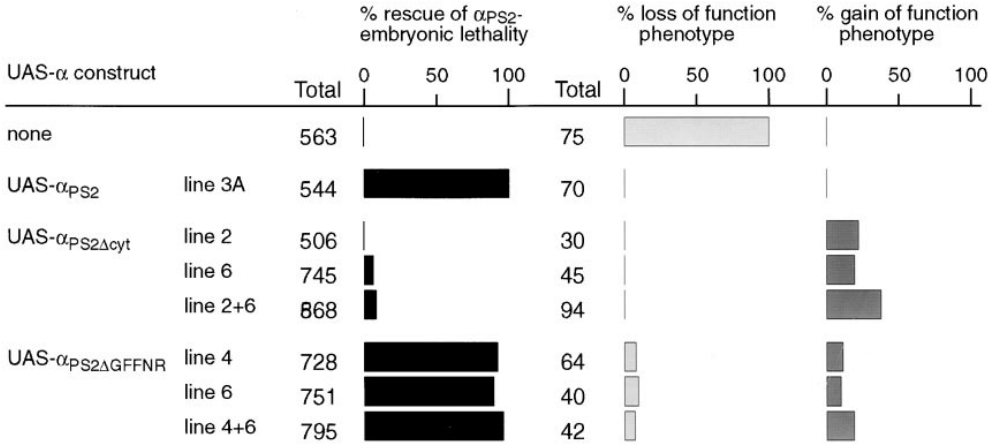


Figure 3. The ability of different combinations of the UAS-α_{PS2} cytoplasmic mutants (driven by twist-GAL4 and 24B) to rescue the α_{PS2} embryonic lethality is indicated by the black bars. The percent rescue of α_{PS2} embryonic lethality was calculated as following: (1/4 total number of embryos − unhatched embryos)/ 1/4 total number of embryos × 100). The total number of embryos counted is shown on the left side of each bar. The embryonic lethality of α_{PS2} mutants can be rescued with either the UAS-α_{PS2} (completely) or the UAS-$\alpha_{PS2\Delta GFFNR}$ (nearly completely) constructs. In contrast, the UAS-$\alpha_{PS2\Delta cyt}$ construct is unable to rescue the lethality. A subset of embryos containing 50% mutant and 50% wild-type embryos was selected using a marked balancer chromosome (the number is indicated to the left side of each grey bar). This subset was examined by staining the muscles for muscle myosin (see Fig. 4), and was scored for a loss of function (muscle detachment, *light grey bars*) or gain of function (formation of ectopic attachment sites, *dark grey bars*) phenotypes. The percent of embryos with muscle phenotype was calculated as follows: (number of embryos with muscle phenotype)/ 1/2 number of selected embryos × 100). Deletion of the cytoplasmic tail, and to a lesser extent deletion of the GFFNR motif, causes gain-of-function phenotypes.

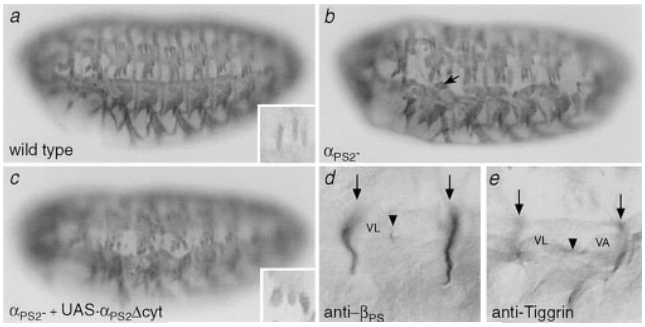


Figure 4. The cytoplasmic domain of the α_{PS2} subunit is required for normal pattern of muscle attachments. Embryos in *a–c* are stained with an antibody for muscle myosin to reveal the pattern of the muscles. In embryos that lack the α_{PS2} subunit, the muscles detach (*arrow*; *b*) compared with wild-type (*a*). Expression of $\alpha_{PS2\Delta cyt}$ in the muscles of mutant embryos rescues the muscle detachment phenotype (*c*). However, the transverse muscles look broader at their tips (see *inset* in *c* and compare with *inset* in *a*), and in 37% of the mutant embryos (Fig. 3) the ventral lateral muscle (*VL*) form ectopic muscle attachments with the ventral acute muscles (*VA*; shown in *c,* and in higher magnification in *d* and *e*). These new attachment sites contain the mutant PS2 integrin (*d*; revealed with an antibody against β_{PS}) and the extracellular PS2 ligand, Tiggrin (*e*). *d* and *e* show one segment with the segment borders marked by arrows.

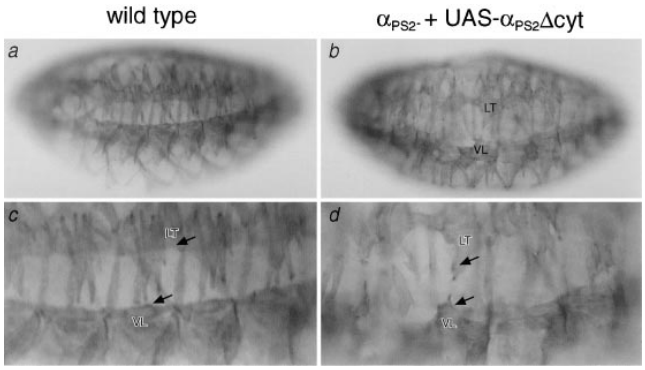


Figure 5. The cytoplasmic domain of the α_{PS2} subunit is required to prevent ectopic processes from the lateral surfaces of the muscles. An antibody against the transmembrane protein gp150 has been used to show the muscle surface. *a* and *b* show the outline of the muscles of a wild-type embryo at two different magnifications. The higher magnification panel shows that wild-type muscles at stage 16 have smooth lateral surfaces. In contrast, muscles that express $\alpha_{PS2\Delta cyt}$ (*c* and *d*) have processes emerging from their lateral surface. *d* shows how ventral (*VL*) and longitudinal (*LT*) muscles in mutant embryos with $\alpha_{PS2\Delta cyt}$ send out growth cone–like processes to each other (*black arrow*).

where many muscles attach (three layers from external to internal). These phenotypes are never observed in *if* mutant embryos carrying the wild-type UAS-α_{PS2} transgene.

The fraction of embryos that have broader tips and ectopic attachment sites varies with the different constructs (Fig. 3). Using one copy of the UAS-$\alpha_{PS2\Delta cyt}$ construct, we find that ~20% of the mutant embryos have both phenotypes (Fig. 3), and two copies increase the fraction to 38% (Fig. 3). Enlargement of the tips of the transverse muscles occurs in all of the segments analyzed (T2–A8). Ectopic attachments or abnormal processes are found in 3.8 segments per embryo on average (from 27 embryos with a phenotype), usually in segments A2–A5. As mentioned before, the UAS-$\alpha_{PS2\Delta GFFNR}$ construct is also able to generate these phenotypes, although less frequently, with only 10% of the embryos having these phenotypes when a single copy of the construct is expressed in them, which increases to 18% when using two copies (Fig. 3). Ectopic attachments or abnormal processes are found in 1.7 segments per embryo (from 31 embryos with a phenotype), while broadening of the tips of the transverse muscles is found in all segments examined.

In summary, PS2 integrins containing mutant α subunit cytoplasmic domains are able to mediate adhesion of the embryonic muscles. However, deletion of the cytoplasmic domain, and to a lesser degree the GFFNR motif, causes enhanced adhesive activity such that the extent of the muscle surface that forms an attachment is increased, and abnormal attachments are formed along the lateral surfaces of the muscles. When the entire cytoplasmic domain is deleted, excessive adhesion by the muscles is so extensive that it causes embryonic lethality. These results suggest that there is a mechanism that normally limits PS2 integrin adhesion to the discrete regions at the ends of the muscles, acting on the cytoplasmic domain of the α_{PS2} subunit. One way this mechanism could be achieved is by inside-out activation of PS2 integrin affinity only at the muscle termini. Excessive adhesion of the muscles is consistent with the possibility that the α_{PS2} subunit cytoplasmic domain deletions cause constitutive activation of the PS2 integrin, converting the integrin to a high-affinity form, as has been shown for the integrin $\alpha_{IIb}\beta_3$ (O'Toole et al., 1994).

Neither of the new phenotypes, larger or ectopic muscle attachments, are observed when we express the mutant transgenes in a wild-type genetic background, demonstrating that this phenotype is only caused in the absence of the endogenous α_{PS2} gene. This observation suggests that if the wild-type α_{PS2} subunit is available, the β_{PS} subunit will form heterodimers with it in preference to the α_{PS2} subunits that have mutant or absent cytoplasmic tails, even when the mutant forms are overexpressed (see below). This suggestion may explain why increasing the number of copies of the UAS-mutant α_{PS2} construct increases the frequency of the phenotype, even when a single copy appears to produce excess α_{PS2} protein. Therefore, heterodimer formation may be normally initiated by interactions between the cytoplasmic domains, as suggested by previous work (Briesewitz et al., 1995).

The other possible explanation of these results is that excessive muscle adhesion only occurs when all the PS2 integrins on the surface are mutants. This explanation suggests a model where wild-type integrin can send signals to downregulate adhesion from the lateral surfaces.

Different Requirements for Inside-out PS2 Signaling in Different Embryonic Tissues

We next tested the requirements for the α cytoplasmic domain in another main site of PS2 integrin function: gut development. We have examined the midgut, the associated gastric caeca, and the proventriculus, which is part of the

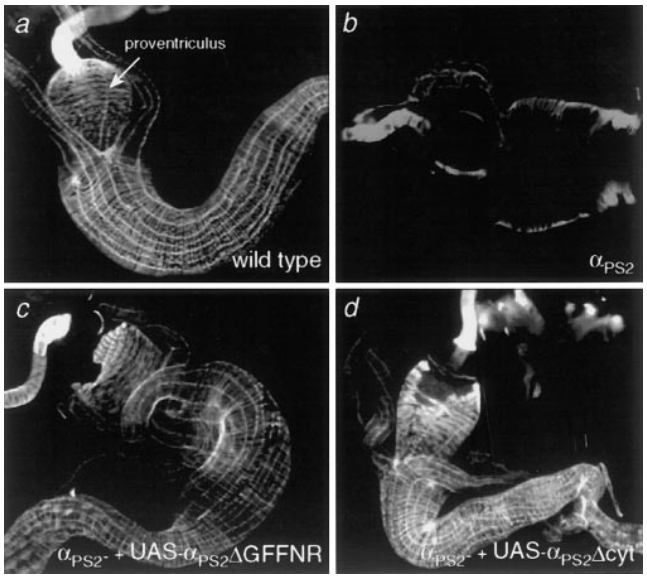


Figure 6. The α_{PS2} cytoplasmic domain is not essential for PS2 integrin function in the visceral mesoderm. Dissected guts stained for actin with phalloidin conjugated to rhodamine show the visceral mesoderm surrounding the gut. The gut musculature is severely disrupted in animals that lack the α_{PS2} subunit (*b*) in contrast to the visceral mesoderm surrounding the gut in wild-type individuals (*a*). In α_{PS2} mutants that carry either the $\alpha_{PS2\Delta GFFNR}$ construct (*c*) or the $\alpha_{PS2\Delta cyt}$ construct (*d*), the visceral mesoderm phenotype is almost completely rescued, with only a mild detachment around the proventriculus remaining.

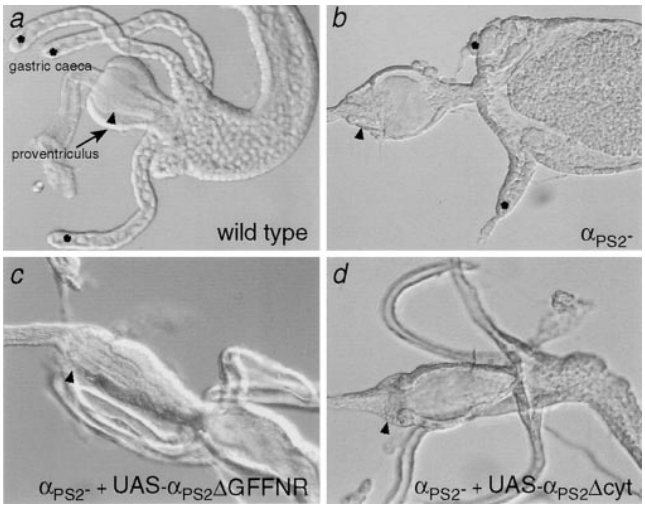
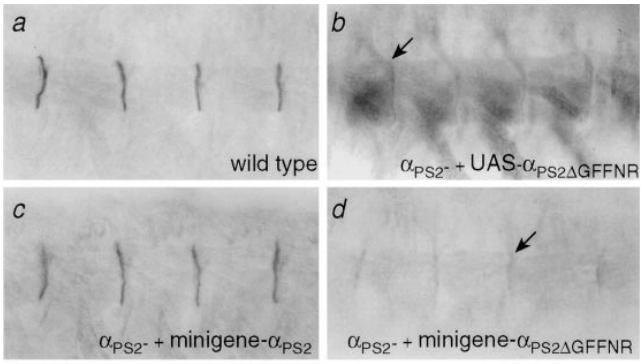


Figure 7. The α_{PS2} cytoplasmic domain is required for PS2 integrin function in the proventriculus, but is not required for morphogenesis of the gastric caeca. At this stage, a wild-type proventriculus is a three-layered valvelike structure at the junction with the midgut. Four long, thin gastric caeca (three of them are marked with dots, and the remaining one is out of the plane of focus) are formed (*a*). Embryos lacking the α_{PS2} subunit show an abnormal proventriculus in which the inner layer has been pulled out, and only two blunt gastric caeca are seen (*dots*; *b*). Both phenotypes are fully rescued by a GAL4-driven wild-type α_{PS2} construct (not shown), but only the gastric caeca, and not the proventriculus, are rescued by $\alpha_{PS2\Delta GFFNR}$ or $\alpha_{PS2\Delta cyt.}$

foregut. There are several phenotypes in these tissues associated with the loss of the PS2 integrin: (*a*) morphogenesis of the gastric caeca does not progress normally, with only two blunt gastric caeca being formed instead of the four long gastric caeca formed in a wild-type larvae (see Fig. 7 and Martin-Bermudo et al., 1997); (*b*) the midgut does not elongate properly (Figs. 6 and 7); and (*c*) the continuity of the visceral mesoderm layer surrounding the gut is disrupted, as seen by phalloidin staining of filamentous actin in the visceral muscles (Fig. 6). Development of the proventriculus is normal until late stage 16 in the absence of the α_{PS2} integrin subunit (Pankratz and Hoch, 1995), but we have found defects later in embryogenesis (Fig. 7): the keyhole region of the esophagus migrates inwards to form the inner layer of the esophagus, but becomes pulled out in PS2 mutant guts (Fig. 7, *a* and *b*; *arrowheads*; Pankratz and Hoch, 1995). This, like the muscle attachments, is an adhesion defect, since the structure initially forms normally (Pankratz and Hoch, 1995). When we express either of the two truncated forms of the α_{PS2} subunit, we observe a complete rescue of the morphogenesis of the gastric caeca and elongation of the midgut (Fig. 6 and 7), as we have seen with the wild-type subunit (Martin-Bermudo et al., 1997). In addition, the visceral mesoderm phenotype is almost completely rescued. It is only at late stage 17 that the visceral mesoderm becomes mildly disrupted at only one position along the gut of embryos carrying the α_{PS2} subunit mutant forms (Fig. 6). Both mutant PS2 integrins fail to rescue the proventriculus phenotype (Fig. 7). Since these phenotypes occur at the very end of embryogenesis, they could be due to the reduced stability of the truncated forms of the α_{PS2} subunit, or to the fact that the GAL4 drivers that we have used do not result in wild-type levels of PS2 integrin expression in these particular tissues, even though the wild-type UAS-α_{PS2} is able to rescue these phenotypes completely. This latter explanation is reinforced by the fact that genetically defined weak (hypomorphic) mutations in the α_{PS2} subunit show identical phenotypes (Bloor and Brown, 1998, and our unpublished observations). We have not observed any gain of function phenotypes in the developing gut.

Does the GFFNR Motif of the α_{PS2} Cytoplasmic Tail Play a Role in Synthesis or Stability of the PS2 Integrin?

In some cases the cytoplasmic tail of the α subunit is essential for effective translation or surface expression of the integrin heterodimer (e.g., Bauer et al., 1993). Our results in the *Drosophila* embryo using the GAL4 system show that deletions within the α_{PS2} cytoplasmic domain do not prevent surface expression of the PS2 heterodimer. However, using the GAL4 system leads to much higher levels of expression of the α_{PS2} subunits compared with the endogenous level of PS2 integrin expression (Fig. 8 *b*), and the excess remains in the endoplasmic reticulum. This overexpression could mask any requirement of the cytoplasmic domain in making a stable PS2 integrin at the end of the muscles. In fact, despite this overexpression there appears to be less PS2 heterodimer at the end of the muscles, with GAL4-expressed mutants compared with the GAL4-expressed wild-type α_{PS2} subunit, judging by the staining



f

construct		total	% rescue of α_{PS2}- embryonic lethality
none		600	0
minigene-α_{PS2}	line 1	940	100
	line 2	620	100
minigene-$\alpha_{PS2\Delta GFFNR}$	line 1	611	0
	line 2	603	0
	line 1+2	500	0

Figure 8. The GFFNR motif is required for wild-type levels of cell surface expression of the PS2 integrin. In this case, expression of PS2 integrins has been detected using an antibody against α_{PS2}. Using the GAL4 system to express the $\alpha_{PS2\Delta GFFNR}$ construct leads to an excess of α_{PS2}. One fraction of it is localized to the attachment sites (*arrow*), and the rest remains inside the cell. *b* shows how α_{PS2} made by a minigene ($minigene_{wt}$) is localized at the surface of the muscles at the attachment sites (*arrow*) at levels indistinguishable from the wild-type α_{PS2} (*a*). In contrast, a deletion of the GFFNR motif within the minigene results in greatly reduced surface expression (*c*).

with an antibody against the β subunit (Fig. 1 and data not shown), although this is hard to quantify. Therefore, to clarify whether deletion of the GFFNR within the cytoplasmic domain affects the ability of the α_{PS2} subunit to form a stable heterodimer with the β_{PS} subunit, we constructed a gene that will express $\alpha_{PS2\Delta GFFNR}$ at levels similar to those of the wild-type gene. To do this we used a shortened version of α_{PS2} gene, a minigene containing 24 kb of the α_{PS2} genomic DNA, that is able to rescue completely the embryonic lethality of an *if* null mutation (Bloor and Brown, 1998). This construct is expressed in the somatic muscles at levels very similar to those of the wild-type α_{PS2} gene (Fig. 8 *c*). Deletion of the GFFNR motif within the α cytoplasmic domain of this minigene leads to very low levels of PS2 expression in the muscles (compare Fig. 8, *d* with *c* and *a*), and it does not rescue the embryonic lethality associated with an *if* null mutation. Independent lines of each construct yielded similar results, ruling out the possibility that the difference in the levels of expression are due to the site of insertion of the transgenes. From these results we conclude that the highly conserved GFFNR motif is indeed required for effective synthesis, assembly, and/or stability of the PS2 integrin at the muscle attachment sites.

Discussion

In this work we have examined the requirements for the integrin α_{PS2} subunit cytoplasmic domain in modulating integrin activity in the developing embryo. We have shown that the cytoplasmic domain is not required for PS2 integrin adhesion, but instead is required to prevent adhesion at the wrong locations. In the wild-type *Drosophila* embryo, PS2 integrin adhesion is tightly localized to specific sites at the ends of the somatic muscles. In contrast, when the wild-type PS2 integrin is replaced with mutant forms of the PS2 integrin lacking portions of the cytoplasmic domain, then PS2 integrin–mediated adhesion is no longer tightly localized. The muscles make ectopic attachments and send out growth cone–like processes, not only from the ends of the muscles, but also from their lateral surfaces. This indicates that we have created a PS2 integrin that is functional around the entire surface of the muscles rather than just at the ends. Therefore, we conclude that the cytoplasmic domain of the α_{PS2} subunit is required to keep the PS2 integrin inactive along the lateral surfaces of the muscles. Normally this inhibition is only released at the ends of the muscles where strong attachment is needed.

Control of PS2 integrin adhesion at the ends of the muscles is part of a multistep process that results in a precise pattern of strong muscle–muscle and muscle–epidermal cell adhesion. This process starts with an initial recognition/adhesion step, where the muscles make their contact with and attach to specific epidermal cells. This first step is independent of integrins and involves formation of short regions of close membrane contact that are not by themselves strong enough to withstand the force of muscle contraction in the absence of integrin adhesion (Prokop et al., 1998). The muscle–epidermis and muscle–muscle attachments then differentiate by forming extensive hemiadherens junctions, and strong PS integrin-dependent adhesion develops. The data we have presented here suggests that part of muscle attachment differentiation is activation of PS2 integrin adhesion, specifically at the ends of the muscles. This activation could be controlled by an inherent intracellular polarity of the muscles that localizes a protein to the ends of the muscles, which activates the PS2 integrin through the cytoplasmic domain of the α_{PS2} subunit. However, it seems more likely that PS2 integrin adhesion should only occur after successful attachment of the muscle to the correct cell, and therefore be triggered by an extracellular signal. This mechanism would avoid the kind of ectopic attachments that we have observed when the PS2 integrin cytoplasmic domain mutants are present in the muscles. The extracellular signal could be transmitted by an unknown transmembrane receptor that sends an intracellular signal, acting on the α_{PS2} subunit cytoplasmic tail to initiate adhesion. It is also possible that the PS2 integrin itself transmits the signal that results in integrin activation. The low-affinity interaction between PS2 and a localized extracellular ligand might convert PS2 into a high-affinity conformation that binds more strongly to a variety of extracellular ligands, including Tiggrin. A similar ligand-dependent activation has been described for the $\alpha_{IIb}\beta_3$ integrin (Du et al., 1991). Interaction of PS2 with ligands could also stabilize the active state at the ends of the muscles, as has been proposed for other integrins (Keizer et al., 1988; van Kooyk et al., 1991). If this last model is true, then deletion of the cytoplasmic tail of the α_{PS2} subunit mimics an activa-

tion that normally occurs by extracellular interaction of a ligand with the integrin.

Our experiments have not determined the mechanistic basis of the activation that occurs when the α_{PS2} cytoplasmic tail is deleted. If the PS2 integrin behaves like the platelet integrin $\alpha_{IIb}\beta_3$, then activation occurs by a conformational change in the protein that increases the affinity of the integrin for its ligands, which may be combined with an increase in avidity promoted by clustering of the integrin. As the cytoplasmic domain mutants of the PS2 integrin are expressed at modestly lower levels than the wild-type PS2 integrin, it seems unlikely that the gain-of-function phenotypes are caused by the mutations, leading to excessive quantities of the integrin, or one that turns over less rapidly. Indeed, the gain-of-function phenotypes do not occur when the PS2 heterodimer is overexpressed by GAL4, driving expression of both UAS-α_{PS2} and UAS-β_{PS} constructs (our unpublished observations). In addition, the process of muscle attachment occurs very rapidly (within 4 h), making changes in the rate of turnover or assembly less likely to contribute to the gain-of-function phenotypes. It is therefore tempting to speculate that, similar to the integrin $\alpha_{IIb}\beta_3$, the affinity of PS2 integrin for extracellular ligands is modulated by the α_{PS2} cytoplasmic domain.

We have also analyzed the effect that deletions within the cytoplasmic tail of the α_{PS2} subunit have on the function of the PS2 integrin in other tissues. In general, the mutant PS2 integrins can replace the wild-type PS2 integrin in the visceral mesoderm and promote normal morphogenesis of the midgut. We have not detected any gain-of-function phenotypes that would indicate that control of the activation state of the PS2 integrin in the visceral mesoderm is as vital as it is in the somatic muscles. This result demonstrates that the different embryonic tissues have different requirements for the α_{PS2} subunit cytoplasmic domain, consistent with previous studies showing that different cell types have different ways of regulating integrin activity. If information processing by the signaling machinery of a cell is dependent on cell type, then one consequence might be for one cell type to possess integrins in a higher activation state than other types. Therefore, there are two possible ways to explain how the α_{PS2} cytoplasmic domain can contribute in different ways to PS2 function: (*a*) there could be tissues in which the PS2 integrin is required to be active all the time, as in the visceral mesoderm, while in other tissues like the somatic muscles its activity needs to be regulated. This difference may reflect the importance for the large multinucleate somatic muscles to have discrete sites of integrin adhesion, while the visceral muscles may have more uniform adhesion along the cell surface. (*b*) the PS2 integrin might perform different functions in the different tissues (e.g. mediating adhesion, migration, or differentiation), and the α_{PS2} cytoplasmic tail might not be essential for all of these cellular functions. This latter hypothesis is consistent with data showing that deletion of the α_5 cytoplasmic domain of the $\alpha_5\beta_1$ integrin still permits efficient adhesion and increases in tyrosine phosphorylation, but causes reduced motility and cell spreading (Bauer et al., 1993). Identification of proteins that interact with the α subunit cytoplasmic domains will provide insight into the mechanisms of this regulation.

Finally, our results have shown that deletion of the entire cytoplasmic domain has a stronger effect than deletion of the highly conserved motif GFFNR, suggesting that there must be other regions within the α_{PS2} cytoplasmic domain that contribute to PS2 integrin function regulation. A more detailed analysis of small deletions within the cytoplasmic domain will help to identify the different regions involved in regulating PS2 integrin function.

In summary, our results show that during embryogenesis it is essential to have a mechanism to regulate integrin function at the right places. We have found that being able to keep integrin adhesion off at the right moment and place is just as important as having active integrins. We also show that a mechanism to regulate integrin activity exists in the embryo, and that activation of the integrin by this mechanism can be mimicked by deletion of the α_{PS2} subunit cytoplasmic tail, suggesting that the mechanism acts on this domain. A further characterization of this mechanism will allow us to test whether it is only required in some tissues during embryogenesis, or whether a similar mechanism is used in different tissues.

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