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 (αPS2βPS) 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 αPS2 with mutant forms containing cytoplasmic domain deletions results in the rescue of the majority of defects associated with the absence of the αPS2 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 αPS2 subunit cytoplasmic domain is required for inside-out regulation of integrin affinity, as has been seen with the integrin αIIbβ3.

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 αIbβ3 integrin, which is already present on the cell surface in an inactive form (Manning and Brass, 1991; Shattil and Brugge, 1991). The β3 integrins expressed on leukocytes undergo activation in a similar way to that occurring in αIIbβ3 integrins (Arnauot, 1990; Larson and Springer, 1990). At sites of inflammation, the β3 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 co-stimulating receptor CD2 leads to activation of α2β3 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 β1 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 β1 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 αIIbβ3 integrin on resting circulatory platelets may lead to thrombosis (Kieffer and Phillips, 1990; Kieffer et al., 1991), and activation of the β3 integrins at the wrong places on leukocytes may lead to inflammation (Arnauot, 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-
Loss of function mutations in inflated (if; αPS2 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 (Babraht 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 βPS integrin subunit is essential during development for βPS 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 PS subdomain has generated a constitutively active αPS2β2 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 αPS2 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-αPS2 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 GFFNKR motif (ΔGFFNKR). To make the Δcyt mutant, we amplified a portion of the αPS2 gene from a subclone of αPS2 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) and one center primer (vector primer sequence), to produce a PCR fragment. This PCR fragment was digested with BglII and SalI, gel-purified and cloned into the UAS vector. The PCR fragment was then used to replace the corresponding fragment in the wild-type UAS-αPS2 construct (Martin-Bermudo et al., 1997). To obtain the ΔGFFNKR construct, the same procedure was followed as for Δcyt but in this case the mutant primer was the bases encoding the GFFNKR motif at the start of exon 11 (5’ GGT GTC CTC AAC TAG GAT CAT TAA CCC TTT CTC 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-αPS2 construct (Martin-Bermudo et al., 1997). To obtain the ΔGFFNKR construct, the same procedure was followed as for Δcyt, but in this case the mutant primer was the bases encoding the GFFNKR motif at the start of exon 12 (5’ TTA ACC CTA CAG TGC AAC CGG CCA ACG GAT CAC TCG C 3’). To generate germline transplants, both constructs were transfected into flies using standard methods.

The construction of the minigene has been described (Bloor and Brown, 1998). To generate the ΔGFFNKR mutant form of this minigene, we replaced a SacII-RsrII fragment in the wild-type minigene with that from the UAS-αPS2ΔGFFNKR.

Drosophila Strains

The integrin mutant allele used in this study is the null allele ifΔ4 (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-αPS2cyt: 3.A (Martin-Bermudo et al., 1997); UAS-αPS2Δcyt: 1, 2, 6; UAS-αPS2ΔGFFNFR: 4, 6; minigene-αPS2: 47, 55; and minigene-αPS2ΔGFFNFR: 76, 96.

**Histology**

Whole mount staining of embryos was performed using standard procedures. The primary antibodies used were the C66G11 mouse mAb against βPS (1:1,000; Brower et al., 1984), the 7A10 PS2 hc/2 rat mAb against αPS2 (U.S. Bogert, 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 Axioshot 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 αPS2 subunit genes containing deletions within the cytoplasmic domain were constructed (Fig. 1). In one construct, we have deleted the entire cytoplasmic domain (αPS2Δcyt), and in the other we removed the αPS2 variant form of the highly conserved domain GFFKR (αPS2ΔGFFNFR; 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 αPS2 subunit by expressing a wild-type αPS2 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 βPS 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 βPS localization to the ends of muscles in mutant embryos by expressing the αPS2 subunit with the GAL4 system. To analyze the role of the cytoplasmic domain of the αPS2 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 αPS2 subunit in the mesoderm of inflated mutant embryos. To examine whether the mutant αPS2 subunits are able to form heterodimers with the βPS that are properly localized, we stained the different transgenic embryos with an anti-βPS 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 βPS localization (Fig. 2 c and data not shown). From these results we conclude that αPS2 subunits containing deletions in the cytoplasmic domain are able to form heterodimers with the βPS 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 activate it. Therefore, it was not simple to predict the consequences that mutating the cytoplasmic domain of the αPS2 subunit would have on embryonic development.

Expression of the αPS2ΔGFFNFR mutant subunit in embryos that lack αPS2 rescues the if mutations embryonic lethality almost as well as the wild-type construct (Fig. 3; Martin-Bermudo et al., 1997). However, if we express the αPS2Δcyt mutant form, we find that it is unable to rescue the lethality caused by the absence of αPS2 (Fig. 3). This result is not due to lower levels of expression of this mutant integrin, since using two copies of the UAS-αPS2Δcyt transgene does not significantly improve the ability of this mutant form to rescue if (Fig. 3). The failure of the Δcyt truncated form of the αPS2 subunit to rescue the inflated embryonic lethality could be due to the fact that deletion of the cytoplasmic domain creates a nonfunctional αPS2 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 αPS2 Subunit has been replaced with the truncated forms of the αPS2 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 αPS2 cytoplasmic domains. The cytoplasmic tail and part of the transmembrane region of αPS2 subunit are shown at the top. Beneath this, the two mutants generated are shown: αPS2Δcyt and αPS2ΔGFFNFR.](image-url)
Figure 2. The αPS2 cytoplasmic domain is not required for localization of the PS2 integrin to the end of the muscles. An antibody against the βPS 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 αPS2 subunit, the βPS staining at the end of the muscles is lost. (c) Expression of the UAS-αPS2Δcyt construct in an αPS2 mutant embryo produces a mutant heterodimer that is correctly localized at the muscle termini.

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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-αPS2 construct completely rescues the muscle phenotype (Martin-Bermudo et al., 1997). if mutant embryos carrying the UAS-αPS2ΔGFNR 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-αPS2Δcyt construct (Fig. 4 c), despite the failure of this construct to rescue embryonic lethality.

Closer examination revealed that both mutant forms of the αPS2 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 integrin 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 αPS2ΔcytβPS integrin is found at the ectopic attachment sites (Fig. 4 d), and the extracellular matrix protein Tigrin (Fogerty et al., 1994), a PS2 ligand, is also recruited (Fig. 4 e). Although staining of both PS2 and Tigrin 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-αPS2 cytoplasmic mutants (driven by twist-GAL4 and 24B) to rescue the αPS2 embryonic lethality is indicated by the black bars. The percent rescue of αPS2 embryonic lethality was calculated as follows: (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 αPS2 mutants can be rescued with either the UAS-αPS2 (completely) or the UAS-αPS2ΔGFNR (nearly completely) constructs. In contrast, the UAS-αPS2Δcyt 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 GFNR motif, causes gain-of-function phenotypes.
muscle (and in 37% of the mutant embryos (Fig. 3) the ventral lateral acute muscles (and; revealed with an antibody against dgrin (PS2) and the extracellular PS2 ligand, Tiggrin (e). d and e show one segment with the segment borders marked by arrows.

where many muscles attach (three layers from external to internal). These phenotypes are never observed in if mutant embryos carrying the wild-type UAS-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-PS2Δcyt 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-PS2ΔGFFNR 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 αPS2 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 αPS2 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 αIIIβ3 (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 αPS2 gene. This observation suggests that if the wild-type αPS2 subunit is available, the βPS subunit will form heterodimers with it in preference to the αPS2 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 αPS2 construct increases the frequency of the phenotype, even when a single copy appears to produce excess αPS2 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
The αPS2 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 αPS2 subunit (b) in contrast to the visceral mesoderm surrounding the gut in wild-type individuals (a). In αPS2 mutants that carry either the αPS2ΔGFFNR construct (c) or the αPS2Δcyt construct (d), the visceral mesoderm phenotype is almost completely rescued, with only a mild detachment around the proventriculus remaining.

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 αPS2 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 αPS2 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 αPS2 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 αPS2 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-αPS2 is able to rescue these phenotypes completely. This latter explanation is reinforced by the fact that genetically defined weak (hypomorphic) mutations in the αPS2 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 αPS2 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 αPS2 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 αPS2 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 αPS2 subunit, judging by the staining.
Discussion

In this work we have examined the requirements for the integrin $\alpha_{PS2}$ 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 $\alpha_{PS2}$ 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 hemiadh- ersions 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 $\alpha_{PS2}$ 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 $\alpha_{PS2}$ 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 $\alpha_{hB3}$ 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 $\alpha_{PS2}$ subunit mimics an activa-

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 $\alpha_{PS2}$. Using the GAL4 system to express the $\alpha_{PS2}$ construct leads to an excess of $\alpha_{PS2}$. One fraction of it is localized to the attachment sites (arrow), and the rest remains inside the cell. b shows how $\alpha_{PS2}$ made by a minigene (minigene$_{a}$) is localized at the surface of the muscles at the attachment sites (arrow) at levels indistinguishable from the wild-type $\alpha_{PS2}$ (a). In contrast, a deletion of the GFFNR motif within the minigene results in greatly reduced surface expression (c).

with an antibody against the $\beta$ 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 $\alpha_{PS2}$ subunit to form a stable heterodimer with the $\beta_{PS}$ subunit, we constructed a gene that will express $\alpha_{PS2}$GFFNR at levels similar to those of the wild-type $\alpha_{PS2}$ gene. To do this we used a shortened version of $\alpha_{PS2}$ gene, a minigene containing 24 kb of the $\alpha_{PS2}$ 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 $\alpha_{PS2}$ gene (Fig. 8 c). Deletion of the GFFNR motif within the $\alpha$ 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 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.
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 αPS2 cytoplasmic tail is deleted. If the PS2 integrin behaves like the platelet integrin αIIbβ3, 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-αPS2 and UAS-βPS 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 αIIbβ3, the affinity of PS2 integrin for extracellular ligands is modulated by the αPS2 cytoplasmic domain.

We have also analyzed the effect that deletions within the cytoplasmic tail of the αPS2 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 αPS2 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 αPS2 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 αPS2 cytoplasmic tail might not be essential for all of these cellular functions. This latter hypothesis is consistent with data showing that deletion of the α5 cytoplasmic domain of the α5β1 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 αPS2 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 αPS2 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.

We would like to thank A. Brand, D. Kiehart, and K. Zinn for providing reagents and fly stocks, and John Overton for technical assistance. We thank A. Gonzalez Reyes, S. Gregory, and Reviewer I for helpful comments on the manuscript.

This work was supported by fellowships from the Spanish Ministerio de Educacion y Ciencia and the European Economic Community to M.D. Martin-Bermudo and a Wellcome Trust Senior Fellowship to N.H. Brown.

Received for publication 1 December 1997 and in revised form 6 February 1998.

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The Journal of Cell Biology, Volume 141, 1998 1080


