Intracellular Signals Direct Integrin Localization to Sites of Function in Embryonic Muscles

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Abstract. In the *Drosophila* embryo, the $\alpha_{PS2}\beta_{PS}$ integrin heterodimer is localized tightly at the termini of the multinucleate muscles where they attach to the $\alpha_{PS1}\beta_{PS}$-containing epidermal tendon cells. Here we examine the basis for $\alpha_{PS2}\beta_{PS}$ integrin subcellular localization. We show that the β_{PS} cytoplasmic tail is sufficient to direct the localization of a heterologous transmembrane protein, CD2, to the muscle termini in vivo. This localization does not occur via an association with structures set up by the endogenous β_{PS} integrins, since it can occur even in the absence of the β_{PS} protein. Furthermore, the subcellular localization of the $\alpha_{PS2}\beta_{PS}$ integrin is not dependent on any other interactions between the muscles and the tendon cells. In embryos that lack the segmental tendon cells, due to a mutation removing the related segment polarity genes *engrailed* and *invected*, $\alpha_{PS2}\beta_{PS}$ is still localized to the muscle termini even though the ventral longitudinal muscles are not attached to the epidermis, but instead are attached end to end. Thus the $\alpha_{PS2}\beta_{PS}$ integrin can be localized by an intracellular mechanism within the muscles. Our results challenge the view that the transmission of signals from the extracellular environment via integrins is required for the organization of the cytoskeleton and the resultant cellular polarity.

INTERACTIONS between cells are essential for the development of multicellular organisms, and depend on plasma membrane proteins that mediate adhesion and signaling through protein–protein and protein–carbohydrate interactions. One essential family of cell surface receptors is the integrins (for review see Hynes, 1992). These are α/β heterodimeric transmembrane proteins that bind to transmembrane and extracellular matrix proteins. The α and β subunits must form a heterodimer to make a functional receptor that is transported to the cell surface (Cheresh and Spiro, 1987; Kishimoto et al., 1987; Leptin et al., 1989). Both α and β subunits contribute to the extracellular ligand–binding region (D'Souza et al., 1990; Smith and Cheresh, 1990), while it is primarily the β cytoplasmic tail that binds to intracellular proteins (LaFlamme et al., 1992). The binding of integrins to their ligands also leads to intracellular changes indicative of signaling such as tyrosine phosphorylation (for review see Clark and Brugge, 1995). Analysis of the phenotypes produced by mutations in genes encoding integrins reveals that many integrin heterodimers are essential for normal development. Thus in *Drosophila*, the defects observed in embryos mutant for the β_{PS} subunit highlight the role of integrins in adhesion between the muscles and the tendon cells. In embryos that lack the segmental tendon cells, due to a mutation removing the related segment polarity genes *engrailed* and *invected*, $\alpha_{PS2}\beta_{PS}$ is still localized to the muscle termini even though the ventral longitudinal muscles are not attached to the epidermis, but instead are attached end to end. Thus the $\alpha_{PS2}\beta_{PS}$ integrin can be localized by an intracellular mechanism within the muscles. Our results challenge the view that the transmission of signals from the extracellular environment via integrins is required for the organization of the cytoskeleton and the resultant cellular polarity.

between cell layers (for review see Brown, 1993). In *Caenorhabditis elegans*, the β_{pat-3} integrin subunit is essential for the development of the muscle sarcomeric structure (Hresko et al., 1994; Williams and Waterston, 1994; Gettner et al., 1995), as are *Drosophila* integrins (Volk et al., 1990). Mutations in the mouse α_5, α_4, and β_1 subunit genes all lead to embryonic lethality (Yang et al., 1993; Fassler and Meyer, 1995; Stephens et al., 1995; Yang et al., 1995). Integrins are also required for blood clotting and lymphocyte function (for review see Hynes, 1992).

In cells in culture integrins are required to form focal adhesions, which are sites of close apposition between the plasma membrane and the extracellular substrate where actin bundles terminate, and a variety of proteins, such as talin, vinculin, and focal adhesion kinase, are enriched (for review see Burridge et al., 1988; Turner, 1994). The formation of focal adhesions appears to proceed from outside the cell inward, starting with integrins binding to extracellular ligands, followed by the organization of cytoskeletal components. This view arises from the following observations: first, focal adhesions are observed only on the surface of the cell that is in contact with the extracellular substrate (Burridge et al., 1988) and require an integrin that binds to a component of the substrate (e.g., Ylanne et al., 1993). Second, integrins must be bound to a ligand to be associated with a focal adhesion. When cells containing two integrins, which bind to different ligands, attach and spread on one of the ligands, only the integrin that binds

to that ligand is found in the focal adhesions while the other integrin is found distributed diffusely over the cell surface (e.g., Fath et al., 1989). Third, when integrins bind to extracellular ligands, they become clustered, which by itself induces increased tyrosine phosphorylation of intracellular proteins (Miyamoto et al., 1995). If this phosphorylation is required for the formation of focal adhesions, then the clustering is another key step initiated by the extracellular ligand. Taken together these experiments suggest that integrins must first bind to an extracellular ligand to either form a focal adhesion or bind to components within a prexisting focal adhesion. The experiments described here are designed to test whether this also occurs in vivo.

In the developing organism, structures most similar to focal adhesions are found at the sites where the muscles attach to epidermal cells (invertebrates) or tendons (vertebrates), and at the dense plaques of smooth muscle (Burridge et al., 1988). In this work we concentrate on muscle attachments in the Drosophila embryo. The somatic muscles attach at their ends to specialized epidermal cells, which are called tendon cells because they link the muscles to the exoskeleton and are therefore analogous to tendons in vertebrates (for review see Bate, 1993). Two integrins are found to have complementary expression patterns at the muscle attachment sites: $\alpha_{PS1}\beta_{PS}$ is expressed on the basal surfaces of the tendon cells, while $\alpha_{PS2}\beta_{PS}$ is expressed at the ends of the muscles where they attach to the tendon cells (Bogaert et al., 1987; Leptin et al., 1989). These integrins are required for the maintenance of muscle attachments but not their initial formation (Wright, 1960; Newman and Wright, 1981; Leptin et al., 1989; Brabant and Brower, 1993; Brown, 1994). In addition, the requirement for the two different integrins on either side of the muscle attachment sites does not appear equivalent, since embryos mutant for the α_{PS1} subunit maintain attached muscles (Brower et al., 1995). This observation, as well as the difference between the α_{PS2} and β_{PS} null phenotypes (Brown, 1994), rules out a possible model where $\alpha_{PS2}\beta_{PS}$ binds directly to $\alpha_{PS1}\beta_{PS}$. It further suggests that the function of $\alpha_{PS2}\beta_{PS}$ integrin in the muscles is particularly critical for the maintenance of attached muscles. However, we do not know what the essential integrin ligands at the muscle attachment sites are; the function of an $\alpha_{PS2}\beta_{PS}$ extracellular ligand, tiggrin (Fogerty et al., 1994), has yet to be tested, and mutations in laminin, an $\alpha_{PS1}\beta_{PS}$ ligand (Gotwals et al., 1994), do not cause widespread muscle detachment (Henchcliffe et al., 1993; Yarnitsky and Volk, 1995). Thus the ligands could be extracellular matrix proteins, transmembrane proteins, or both. The ligands could become localized first, leading to integrin localization, or vice versa. Therefore an examination of how the integrins are localized will increase our understanding of how the integrins function at the muscle atttachment site.

Two opposing models for how the $\alpha_{PS2}\beta_{PS}$ integrin functions in the muscles to form a stable muscle attachment can be formulated: an outside-in model and an inside-out model (see Fig. 1). An extrapolation of the apparent role of integrins in forming focal adhesions leads to an outside-in model. In this model the tendon cells provide a spatially restricted extracellular ligand for $\alpha_{PS2}\beta_{PS}$, either through expression of a cell surface ligand or, as shown in the fig-

ure, expression of receptors for secreted ligands. The binding of $\alpha_{PS2}\beta_{PS}$ integrin to these ligands localized by the tendon cells would lead to the accumulation of $\alpha_{PS2}\beta_{PS}$ at the ends of the muscle, where it would organize the muscle cytoskeleton. In the counter model (inside-out), the reverse occurs, in that $\alpha_{PS2}\beta_{PS}$ is localized by intracellular signals in the already polarized muscle. The localized $\alpha_{PS2}\beta_{PS}$ would concentrate extracellular ligands required for muscle attachment and may subsequently play a role in connecting the contractile apparatus to the muscle termini. The inside-out model is similar in part to the mechanism of inside-out activation of the (LFA-1) integrin where clustering of $\alpha_L\beta_2$ by the cytoskeleton increases the avidity of the interaction with ligand (for review see Lub et al., 1995).

We have examined the role of integrins in the formation of "in vivo focal adhesions," by analyzing how $\alpha_{PS2}\beta_{PS}$ is localized to the developing muscle attachments of the Drosophila embryo. We find that chimeras containing the β_{PS} cytoplasmic tail become localized to the ends of the muscles with the endogenous integrins, as is the case in vertebrate focal adhesions. Unexpectedly, however, we find that this specific localization occurs even in the absence of the endogenous integrins. This result, and others we report here, strongly suggest that there is significant inside-out function of integrins in vivo.

Materials and Methods

Preparation of the Modified Integrin Genes

The chimeric UAS-CD2/β_{PS} genes were constructed using the UAS-CD2 gene (Dunin-Borkowski and Brown, 1995) and a cDNA clone encoding β_{PS} (Grinblat et al., 1994). The CD2/β_{PS} (C$\beta\beta$) construct, which encodes the extracellular domain of the CD2 protein and the transmembrane domain and cytoplasmic tail of β_{PS} (see Fig. 2), was made by replacing the HincII-SalI fragment containing the CD2 transmembrane domain and cytoplasmic tail with a HincII-SalI PCR fragment. This PCR fragment was made using an oligonucleotide that fuses CD2 and β_{PS} sequences at the start of the transmembrane domain (see Fig 2; cb1: GAA GTT GTC AAC TGT CCA GAG AAG GTT TTC ATG TTG GGC ATC; underlined residues are β_{PS} sequence) and an oligonucleotide that will introduce a SalI site 33 nt downstream from the β_{PS} stop codon (cb2: GAC AGT CGA CAT AAT TCC CTA ATG TTT AGT T). The CD2/β_{PS} (CCβ) construct, which encodes the extracellular and transmembrane portions of the CD2 protein and the cytoplasmic tail of β_{PS} (Fig. 2), was created by introducing an NdeI site at the junction point in both CD2 and β_{PS} by PCR (CD2: using a primer in the vector and cn:TCC TCT TGC ATA TGC AGA AAA TA, β_{PS}: using βn:CTG TGG AAG CTC ATA TGC ACG ATC CAC GAT and cb2 described above). A HincII to NdeI fragment of the CD2 PCR product and an NdeI to SalI fragment of the β_{PS} PCR product were combined to replace the HincII to SalI fragment of UAS-CD2. Both constructs were introduced into the Drosophila germ line by P element–mediated transformation, and multiple independent transformants were obtained and analyzed. Four independent lines for each construct were examined and produced proteins that were localized, although the levels varied. To consistently detect the CD2/β_{PS} (C$\beta\beta$) protein we had to use both GAL4 drivers, 24B and twist-GAL4, while for the the CD2/β_{PS} (CCβ) protein, the 24B GAL4 line gave sufficently high expression to detect the chimera. Thus it appears that the CD2/β_{PS} (C$\beta\beta$) protein is relatively unstable.

The UAS-β_{PS} gene was constructed from P[mys+] (Grinblat et al., 1994), which contains the genomic sequence for β_{PS}, with an HSP70 polyA addition site in place of the natural polyA site. The GAL4-dependent promoter from pUAST (Brand and Perrimon, 1993) was fused to the first exon of the β_{PS} gene, which is noncoding. Thus the construct contains an SphI to Cel2 (filled in and linked to a SacII site) fragment from pUAST followed by a SacII-SpeI and SpeI-EcoRI fragments from P[mys+],

cloned into a P element vector containing a *white* minigene as a selectable marker (pWhiteRabbit, Brown, N.H., unpublished data).

Drosophila Strains

The alleles used in this study are the null integrin mutants mys^{XG43} and if^{B4} (Leptin et al., 1989; Bunch et al., 1992; Brown, 1994), and $Df(2R)enE$ (Hidalgo, 1994). The mesodermal GAL4 enhancer trap lines are *twist-GAL4* (Greig and Akam, 1993) and 24B (Brand and Perrimon, 1993). The enhancer trap insertion in the *stripe* locus was a gift of Bob Holmgren (Northwestern University, Evanston, IL), and expresses nuclear β-galactosidase starting at stage 12, in a pattern identical to the late expression of the *stripe* enhancer trap lines described in Volk and VijayRaghavan (1994). We confirmed that this enhancer trap is in the *stripe* locus, because the insertion causes a mutation that fails to complement the *stripe^{16}* allele.

Antibody Staining

Whole mount staining of embryos was performed using standard procedures. The primary antibodies used were anti-CD2 OX-34 mAb ascites (1:2,500-1:5,000) (Williams et al., 1987), the CF6G11 mouse mAb against β$_{PS}$ (1:1,000) (Brower et al., 1984), antimuscle myosin (Kiehart and Feghali, 1986) and anti-β-galactosidase (Cappel Laboratories, Malvern, PA). We used either a HRP-linked secondary antibody or a biotin-labeled secondary antibody followed by the Vectastain Elite ABC kit (Vector Labs, Inc., Burlingame, CA) enhancement to stain the embryos. Photographs of stained embryos were taken using a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) and the negatives scanned with a Coolscan (Nikon Inc., Instrument Group, Melville, NY). The scanned images were assembled using Photoshop 3.0 (Adobe Systems, Inc., Mountain View, CA) and labels and drawings added in FreeHand 5.0 (Macromedia, San Francisco, CA).

Results

Does a Heterologous Transmembrane Protein Containing the β$_{PS}$ Cytoplasmic Tail Colocalize with Endogenous Integrins at the Muscle Termini?

It has been shown previously in vertebrate cell culture that chimeric proteins containing the cytoplasmic tail of integrin β subunits fused to heterologous transmembrane proteins are able to colocalize with endogenous integrins at sites of cell–substrate adhesion, or focal adhesions (Geiger et al., 1992; LaFlamme et al., 1992). To test whether this is also true for the β$_{PS}$ subunit in *Drosophila* muscle attachments, we examined the expression of similar chimeric proteins in embryonic muscles. We have shown previously that rat CD2, a member of the immunoglobulin superfamily, can be expressed on the surface of *Drosophila* embryonic muscles and detected with a monoclonal antibody (Dunin-Borkowski and Brown, 1995). This expression of CD2 in the muscles has no effect on normal development, but we were concerned that chimeras of CD2 and the β$_{PS}$ subunit might function as dominant negative proteins and cause lethality if expressed constitutively in the *Drosophila* musculature. Therefore we made use of the GAL4 system (Brand and Perrimon, 1993) to conditionally express our proteins.

We prepared genes encoding two different CD2/β$_{PS}$ chimeras under the control of the UAS$_G$ promoter, which requires the yeast transcription factor GAL4 for expression. After P element–mediated transformation, expression of the chimeric proteins was induced using specific GAL4-expressing lines. Mesodermal expression throughout embryogenesis was achieved using a combination of two different GAL4 lines: (*1*) the enhancer trap line 24B, where a transgene encoding GAL4 has inserted near an enhancer that drives expression in the embryonic mesoderm (Brand and Perrimon, 1993); and (*2*) a gene construct, *twist-GAL4*, composed of the promoter of the mesoderm-specific *twist* gene driving the expression of GAL4 (Greig and Akam, 1993). Since expression of the CD2/β$_{PS}$ chimeras is induced only in the progeny of the crosses between UAS-CD2/β$_{PS}$ and *twist-GAL4*; 24B flies, we are able to examine the localization of CD2/β$_{PS}$ in the embryo even if its expression causes lethality. Thus far the chimeric proteins have not resulted in any dominant negative effects in the wild-type fly (data not shown).

Two chimeric genes were created from the control construct UAS-CD2: the first contains the extracellular domain of CD2 fused to the transmembrane domain and cytoplasmic tail of the β$_{PS}$ subunit (Cββ) and the second contains the extracellular and transmembrane domains of CD2 fused to the cytoplasmic tail of β$_{PS}$ (CCβ; see Fig. 2). The two chimeras and unmodified CD2 were expressed in the muscles of wild-type embryos and detected with a monoclonal antibody to the extracellular portion of CD2. The expression of these three proteins and wild-type β$_{PS}$ containing integrins is shown in Fig. 3. The pattern of attachment sites of the 30 muscles per segment is complex, consisting primarily of sites along the segment boundary where the longitudinal, oblique, and acute muscles attach, but also including spots within each segment where the transverse muscles attach (Armand et al., 1994). For simplicity we have focused on the easily identifiable attachment site at the ventrolateral part of the segment border where the ventral longitudinal muscles attach. In the figures we show two views: an optical horizontal section showing these muscles attached to the epidermis (see Figs. 3 and 5, *a* and *b*) and a view looking through the lateral surface of the epidermis at the muscles (see Figs. 4, 5 *c–h*, and 6). The heterologous protein CD2 is found to be expressed on the entire surface of the muscles (Fig. 3 *a*), while the two CD2/β$_{PS}$ chimeras are found to be localized to the termini of the muscles (Fig. 3, *c* and *d*), in a pattern identical to the wild-type β$_{PS}$ subunit (Fig. 3 *b*). We find that there is more cytoplasmic staining of CD2 and the two chimeric proteins compared with β$_{PS}$, suggesting that they are less efficiently transported to the cell surface. Taking this into account, the CD2/β$_{PS}$ chimeras that reach the plasma membrane appear to be localized to the ends of the muscles as efficiently as β$_{PS}$. Thus, as in mammalian cells, the cytoplasmic tail of the β$_{PS}$ subunit contains sufficient information to direct the localization of transmembrane proteins to sites of integrin function.

Are the Endogenous Integrins Required for the Localization of Chimeric Proteins Containing the β$_{PS}$ Cytoplasmic Tail to the Muscle Termini?

In vertebrate cells chimeric proteins containing the cytoplasmic tail of β subunits become localized to focal adhesions formed by the endogenous integrins (LaFlamme et al., 1992, 1994). To test whether α$_{PS2}$β$_{PS}$ function is required in the muscles for the localization of the CD2/β$_{PS}$ chimeras to the termini, we examined the localization of CD2 and the CD2–integrin chimeras in the muscles of embryos that lack the endogenous integrins. We found that
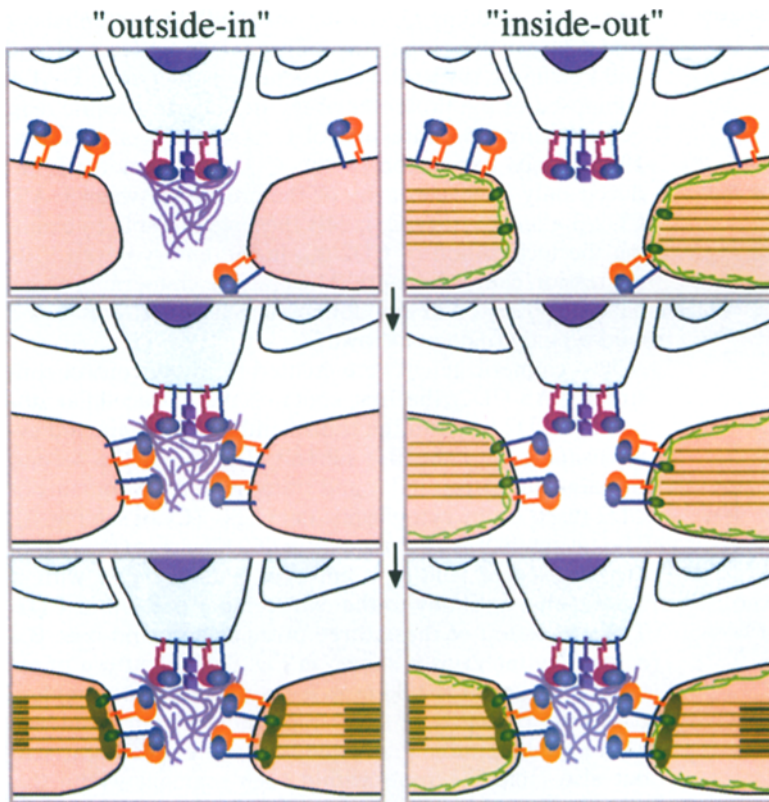
Figure 1. Possible models for $\alpha_{PS2}\beta_{PS}$ integrin function in muscle development. Each panel shows two pink muscles attaching to a central pale blue epidermal tendon cell, and the panels proceed in developmental sequence from top to bottom. The specialization of the tendon cell is indicated by the expression of stripe (*purple nucleus*), the $\alpha_{PS1}\beta_{PS}$ integrin (*blue and red*), and a second low affinity receptor for extracellular matrix proteins (*two purple cubes on a stick*). In the outside-in model, tendon cell receptors lead to the accumulation of extracellular matrix proteins (*purple noodles*), including an $\alpha_{PS2}\beta_{PS}$ ligand. By binding to this ligand, the $\alpha_{PS2}\beta_{PS}$ integrin (*blue and yellow*) is concentrated at the ends of the muscles, where it recruits and organizes cytoskeletal proteins (*green*). In the inside-out model, cytoskeletal components (which may be actin fibers and/or the cortical actin or microtubule based cytoskeleton) lead to the localization of the $\alpha_{PS2}\beta_{PS}$ integrin to the ends of the muscles, where it leads to the accumulation of the extracellular matrix at the muscle attachment site. Even in the inside-out model, the integrin may have some role in the stabilization of the sarcomeric muscle structure (indicated by the addition of cytoskeleton components).

both C$\beta\beta$ (not shown) and CCβ chimeric proteins (Fig. 4 *d*) are still localized to the tips of the muscles in embryos mutant for the α_{PS2} subunit, before their detachment due to loss of α_{PS2} function. To rule out the possibility that the localization of the chimeras arises by residual activity of the β_{PS} subunit (through the formation of a heterodimer with an unknown α), we also examined CD2/β_{PS} localization in muscles of embryos mutant for β_{PS} as well as α_{PS2} (Fig. 4 *f*). The chimeric proteins are still localized to the tips of the muscles, even though the muscles have begun to detach due to the loss of integrin function. Thus the chimeras are not becoming localized simply by binding to proteins assembled by the endogenous $\alpha_{PS2}\beta_{PS}$ integrin. Therefore the cytoplasmic tail of the β_{PS} subunit contains sufficient information to direct the localization of a transmembrane protein to the tips of the embryonic muscles independent of the function of the PS integrins. It has been shown previously that the initial muscle pattern is formed normally in embryos lacking the β_{PS} subunit (Wright, 1960; Newman and Wright, 1981; Leptin et al., 1989), indicating that any cytoskeletal organization or cellular polarity required for this process does not require integrin function. Here we have shown that the assembly of further specialized ends, as revealed by the localization of an integrin, is also not dependent on the function of the integrins.

Since in the outside-in model the muscle polarity requires integrin function, this result suggests that this model is incorrect. However it is presently impossible to rule out the existence of another integrin (as yet unidentified) that is organizing the muscle cytoskeleton in the absence of $\alpha_{PS2}\beta_{PS}$. Nevertheless, since the outside-in model relies on the localization of integrin ligands by the epidermal tendon cells, this model can be tested further by examining the role of epidermal tendon cells in the generation of muscle polarity, as indicated by the localization of $\alpha_{PS2}\beta_{PS}$.

Does Integrin Localization to the Ends of the Muscles Require Signaling from the Epidermal Tendon Cells?

In the outside-in model, $\alpha_{PS2}\beta_{PS}$ becomes localized by binding to a localized extracellular ligand, and then it generates the polarity of the muscle. Before the organization of internal polarity within the muscles, the localized ligand cannot come from the muscles, since an $\alpha_{PS2}\beta_{PS}$ ligand on the muscle surface would lead to integrin accumulation wherever muscle cells contact each other, not just at the ends. This pattern of accumulation is seen for a homophilic cell adhesion molecule, connectin (Nose et al., 1992), which is functionally equivalent to having both $\alpha_{PS2}\beta_{PS}$ and its ligand expressed on the muscle surface, yet

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CD2          EVVNCPEKGLPLYLIVGVSAGGLLLVFFGALFIFCICKRKKRNRRRKGEELEIKASRMSTVERGPKPHSTQASAPASQNPVAS+70aa
β_PS         ENKECPAKVFMLGIVMGVIAAIVLVGLAILLLWKLLTTIHDRREFARFEKERMNAKWDTGENPIYKQATSFKNPMYAGK
CD2/β_PS(Cββ) EVVNCPEKVFMLGIVMGVIAAIVLVGLAILLLWKLLTTIHDRREFARFEKERMNAKWDTGENPIYKQATSFKNPMYAGK
CD2/β_PS(CCβ) EVVNCPEKGLPLYLIVGVSAGGLLLVFFGALFIFCICTIHDRREFARFEKERMNAKWDTGENPIYKQATSFKNPMYAGK
                      transmembrane                cytoplasmic
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Figure 2. Amino acid sequences at the junctions of the chimeric integrin proteins, C$\beta\beta$, and CCβ. The β_{PS} amino acids are bold.
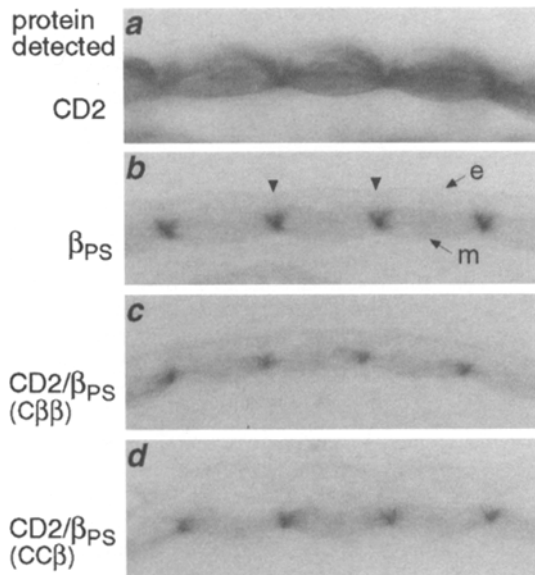
220

Figure 3. The β_{PS} cytoplasmic tail drives the heterologous transmembrane protein CD2 to the ends of the muscles. Horizontal optical sections of anti-CD2 antibody–stained embryos containing the *twi-Gal4* and the *24B-GAL4* genes plus UAS-CD2 (*a*) or UAS-CD2/β_{PS} (C$\beta\beta$) (*c*) or UAS-CD2/β_{PS} (CCβ) (*d*). The CD2-β_{PS} chimeras express at the muscle attachment sites in the same pattern as the β_{PS} protein in wild-type embryos (*b*). In *b* the e indicates the epidermis, with the tendon cells shown by arrowheads, and the ventral longitudinal muscles are indicated with an m.

$\alpha_{PS2}\beta_{PS}$ does not accumulate in this pattern. Therefore if the outside-in model is correct, the ligand must be localized by the epidermal tendon cells. The specification of the pattern within each segment of the epidermis in the embryo is under the control of the segment polarity genes, such as *wingless* and *engrailed*, which also specify the ten-

don cells (Volk and VijayRaghavan, 1994). The majority of muscles attach at the segment border. Although epidermal cells on both sides of the segment boundary are elongated by the attachment of the muscles, it is the cells expressing engrailed at the posterior edge of each segment that form the tendon cells (Fig. 5 *a*). The tendon cells can be distinguished by their expression of markers such as nuclear β-galactosidase produced from an enhancer trap insertion into the *stripe* gene (Volk and VijayRaghavan, 1994; see Fig. 5, *b*, *c*, and *e*).

In embryos missing the genes *engrailed* and its adjacent related gene *invected*, the tendon cells at the posterior margin of each segment are not specified, as indicated by the aberrant pattern of *stripe* nuclear β-galactosidase (Fig. 5, *d*, *f*, and *h*). The pattern of muscles is also disrupted, due to the altered pattern of the epidermis since engrailed does not appear to be required in the mesoderm (Lawrence and Johnston, 1984). If we examine the localization of the $\alpha_{PS2}\beta_{PS}$ integrin in the muscles of these embryos we find that it is still tightly localized to the ends of the muscles (Fig. 5 *h*). In the embryo shown in Fig. 5, *f* and *h*, we can see that the localization occurs even in muscle termini that are not near any tendon cells (*arrowhead*, Fig. 5, *f* and *h*), indicating that the localization can occur without any signals from the tendon cell. Those cells that continue to express *stripe* nuclear β-galactosidase in the *engrailed, invected* mutant embryos do not seem to produce signals for the attachment of the longitudinal muscles, since we do not observe any preference for the muscles to line up with these cells, nor do the muscles underneath these cells have more prominent integrin localization. What can also be observed in these embryos is that even in the absence of segmental border tendon cells the muscles are still attached end to end, indicating some specific adhesion between muscle ends, possibly promoted by the $\alpha_{PS2}\beta_{PS}$ integrin. Thus extracellular signals from the tendon cells are not required to localize integrins to the ends of the mus-
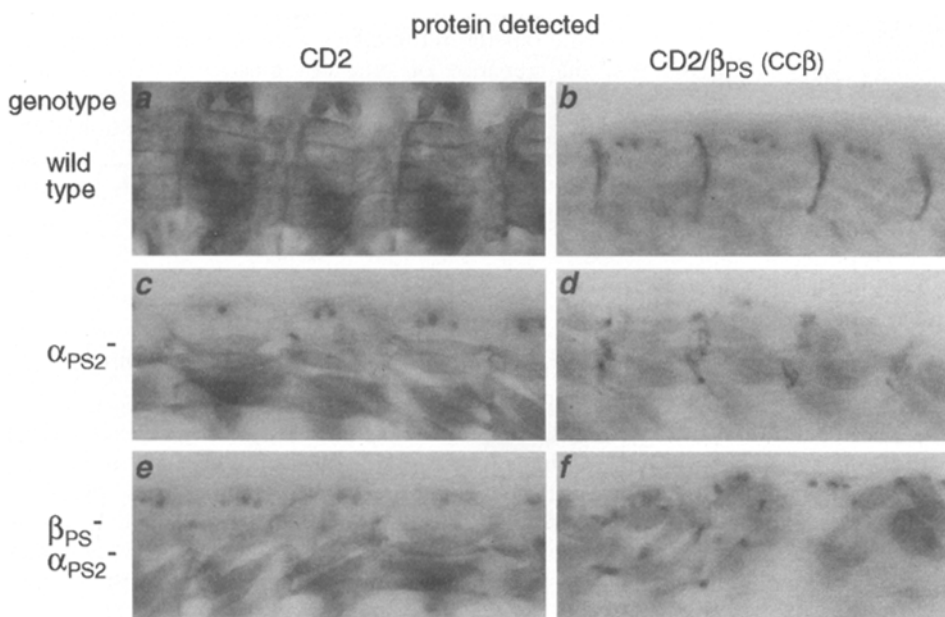

Figure 4. Localization of CD2/β_{PS} chimeric proteins does not require the endogenous integrins. Ventro-lateral views of the expression of CD2 and the CD2/β_{PS} (CCβ) chimera in wild-type embryos (*a* and *b*) and embryos mutant for the α_{PS2} subunit (*c* and *d*), or for both β_{PS} and α_{PS2} subunits (*e* and *f*). All embryos are stained with anti-CD2 antibody. The chimera localizes at the muscle attachment sites even in the absence of endogenous integrins (*d* and *f*). In *b–f* the dots seen toward the top of each panel, just underneath the epidermis, are the transverse muscles in optical section. Embryos are at stage 16, before detachment of the muscles in the embryo mutant for α_{PS2}, and at the start of detachment in the embryo mutant for both β_{PS} and α_{PS2} subunits. Anterior is to the left, dorsal at the top.
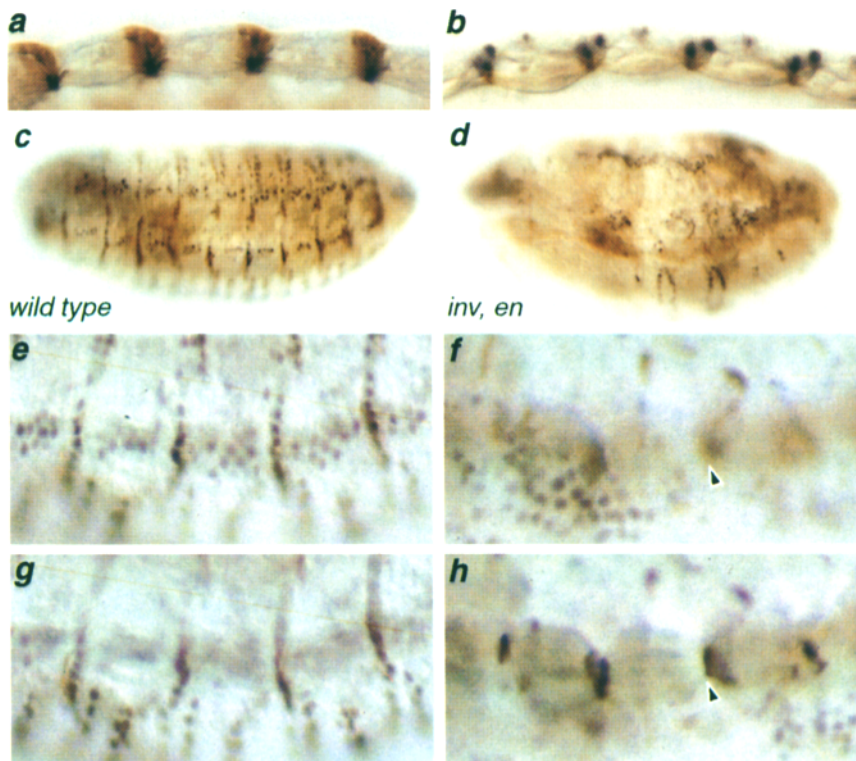
Figure 5. Localization of integrins to the ends of the muscles does not require interaction between the muscles and the tendon cells. *a* and *b* are horizontal optical sections while *c–h* are lateral views. The ventral attachment sites at the segmental border are engrailed-expressing cells, as indicated by their expression of *engrailed-lacZ* (*a*; in brown, with the α_{PS2} subunit stained in black). These cells also express the tendon cell marker produced by an enhancer trap insertion in the *stripe* gene (*b* and *c*; *stripe* nuclear β-galactosidase in black, and $\alpha_{PS2}\beta_{PS}$ with an antibody against the α_{PS2} subunit in brown), as do other tendon cells that are not in the engrailed domain. To examine the effect of altering the fate of these tendon cells on the localization of $\alpha_{PS2}\beta_{PS}$ we examined wild-type embryos (*c*, *e*, and *g*) and embryos mutant for the genes *invected* and *engrailed* (*d*, *f*, and *h*) stained for *stripe* nuclear β-galactosidase (*black*) and α_{PS2} (*brown*). Whole embryos show the disruption in the pattern of *stripe* expression (*c* vs *d*). In dissected embryos, close-up views in two different focal planes show that in the wild-type embryo, *stripe* nuclear β-galactosidase–expressing cells (*e*) overlie the localized $\alpha_{PS2}\beta_{PS}$ integrin (*g*); while in the mutant embryos, even in those areas where there are no *stripe* nuclear β-galactosidase–positive cells in the overlying mutant ectoderm (*f*; arrowhead), localized $\alpha_{PS2}\beta_{PS}$ integrin can be detected at the ends of the muscles (*h*). Embryos are stage 16, anterior is to left.

cles. Instead these results support the inside-out model and indicate that the muscles have an internal polarity which makes the ends different from the lateral surfaces, one facet of which is the localization of $\alpha_{PS2}\beta_{PS}$ to the ends of the muscle.

A Rationale for Internally Directed Integrin Localization: Integrins Are Required Only in the Muscles to Mediate Muscle Attachment

Why is there a mechanism for localizing the integrins to the ends of the muscles that is independent of tendon cells signals? One possibility is that the integrins are primarily required on the muscle side of the attachment, where the tight localization of $\alpha_{PS2}\beta_{PS}$ to the muscle termini is required for the localized assembly of extracellular matrix proteins that are essential for muscle attachment. This is consistent with the fact that the effect of removing the integrin on one side of the attachment site is not equivalent to removing the integrin on the other: in embryos missing the epidermally expressed α_{PS1} subunit the muscles remain attached, while in embryos missing the mesodermally expressed α_{PS2} subunit the muscles detach (Brabant and Brower, 1993; Brown, 1994; Brower et al., 1995). However an alternative interpretation is that there is an additional α subunit that forms a heterodimer with β_{PS} in the epidermis, which complements the loss of the α_{PS1} subunit. To distinguish between these two possibilities, we created embryos that only express the β_{PS} subunit in the mesoderm. This was achieved by constructing a β_{PS} gene under the control of the GAL4-dependent UAS$_G$ promoter, UAS-

β_{PS}, and using the 24B GAL4 line described above to express the β_{PS} subunit in the mesoderm of embryos mutant for the endogenous β_{PS} gene. In these embryos, the muscle detachment phenotype caused by the loss of the β_{PS}-containing integrins is rescued (Fig. 6). Embryos missing the β_{PS} subunit have another phenotype, a defect in the dorsal closure of the epidermis that is independent of the α_{PS2} subunit (Brabant and Brower, 1993; Brown, 1994). As expected, this dorsal hole is not rescued by the restoration of integrin function in the mesoderm (Fig. 6). We can therefore rule out the redundancy argument and demonstrate that maintenance of attached muscles in the embryo mainly requires integrin function in the mesoderm. Thus the internal muscle polarity leads to the localization of the $\alpha_{PS2}\beta_{PS}$ integrin at the ends of the muscle, which is likely to be essential for the maintenance of properly attached muscles.

Discussion

Inside-out Localization of Integrins in the Embryo

Our studies on the localization of $\alpha_{PS2}\beta_{PS}$ integrin to the termini of the embryonic muscles have produced several unexpected results. First, even in the absence of endogenous integrins, chimeric proteins containing the cytoplasmic tail of the β_{PS} subunit are localized to the ends of the muscles. Second, although one might have thought that muscle polarity arises through interactions with the tendon cells, in the absence of the segmental border tendon
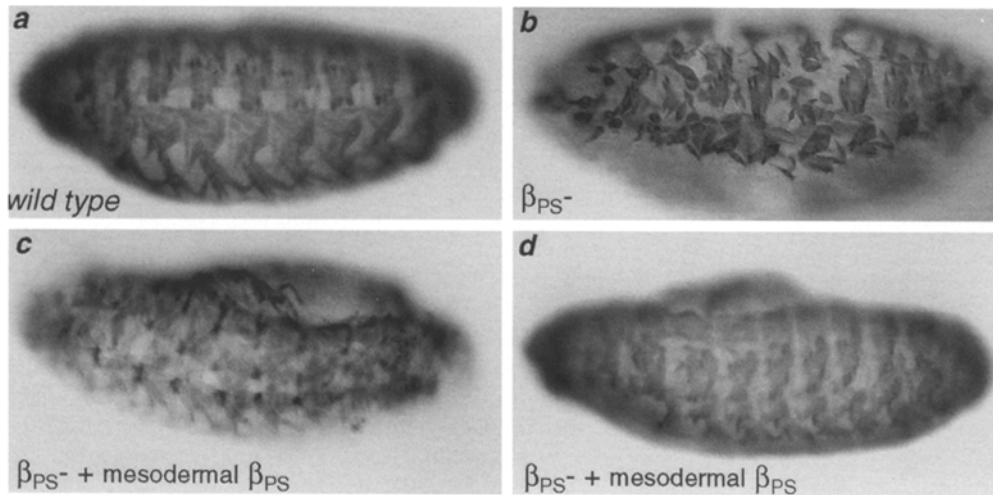
Figure 6. The muscle detachment phenotype of β_{PS} mutant embryos can be rescued by putting back β_{PS} just in the muscles. To show the pattern of muscles the embryos are stained with an antibody to muscle myosin in *a*, *b*, and *d*. In embryos that lack the β_{PS} subunit (*b*), the muscles detach and round up compared with wild-type (*a*). Using the GAL4 system β_{PS} is expressed only in the mesoderm of the mutant embryos, as shown by staining for the β_{PS} subunit (*c*), and this rescues the muscle detachment (*c* and *d*) although not the dorsal hole. Embryos are stage 16, anterior is to left, dorsal up.

cells the muscles that normally attach to these tendon cells are still polarized, as revealed by the localization of $\alpha_{PS2}\beta_{PS}$ integrin and the attachment of these muscles end-to-end. These results demonstrate that in the developing embryo an intracellular mechanism exists to ensure the localization of integrins to their sites of function, contrary to predictions from studies on the formation of focal adhesions in cell culture. Finally, despite the complementary expression of the two integrins $\alpha_{PS1}\beta_{PS}$ and $\alpha_{PS2}\beta_{PS}$, in the epidermis and muscles, respectively, β_{PS}-containing integrins are required only in the muscles for the formation of stable muscle attachments. This suggests that the localization of $\alpha_{PS2}\beta_{PS}$ by the muscles to their ends leads to the localization of specific extracellular matrix proteins, which are essential for muscle attachment.

A comparison between the focal adhesion and the muscle attachment reveals similarities and differences between these two model systems of integrin function. Both are sites of local concentration of integrins and actin fiber insertion into the membrane. However, in most muscles only two sites are formed, while many focal adhesions are formed in a cell spread on a substrate. Since our results, that the localization of chimeric CD2/β_{PS} proteins can occur in the absence of integrin function, are in direct contrast to the situation in cells spread on a substrate, it looks as if that different mechanisms must drive integrin localization in these two systems. This could reflect a functional difference between these two sites of integrin concentration. Alternatively, it is possible that the apparent difference results from the experimental approaches used to analyze integrin localization. Signals from both inside and outside the cell may be important in localizing integrins in both of these model systems. Thus in *Drosophila*, localized ligands may also be able to direct the localization of $\alpha_{PS2}\beta_{PS}$, even if they are not essential. For example, tiggrin, an $\alpha_{PS2}\beta_{PS}$ extracellular ligand, is localized to muscle attachments even in the absence of β_{PS}-containing integrins (Fogerty et al., 1994), so there must be another receptor for tiggrin. If this receptor is expressed by the tendon cells, then $\alpha_{PS2}\beta_{PS}$ could also become localized by

binding to tiggrin, independent from its localization by intracellular signals. This could be tested by examining the ability of integrins lacking the cytoplasmic tails to become localized. Similarly it is possible that in vertebrate cells, the actual site of focal adhesion formation is specified or biased by the cytoskeleton and integrin clustering is initiated by interactions with the cytoskeleton before ligand binding. Although we are not aware of experiments supporting this, in lymphocytes the integrin $\alpha_L\beta_2$ (LFA-1) becomes concentrated at multiple sites by intracellular mechanisms (see Lub et al., 1995 for references). This integrin changes from a low affinity to a high affinity form in response to intracellular signaling. The increase in affinity is composed of two components, first an increase in avidity by clustering $\alpha_L\beta_2$, and second a conformational change that increases the affinity.

As has been found for $\alpha_L\beta_2$, in the developing musculature the clustering of $\alpha_{PS2}\beta_{PS}$ to the ends of the muscles will increase the avidity of receptor–ligand interactions. We do not know whether the $\alpha_{PS2}\beta_{PS}$ integrin also undergoes conformational changes that result in an increased affinity. Nonetheless the tight localization of $\alpha_{PS2}\beta_{PS}$ to the ends of the muscle is an effective form of inside-out signaling analogous to one step of the signaling in lymphocytes. Such a mechanism gives the muscles intracellular control of the initiation of integrin-mediated adhesion by specifying when the dispersed integrin molecules become concentrated at the muscle termini.

The idea that the localization of $\alpha_{PS2}\beta_{PS}$ reflects an intracellular mechanism that allows the muscles to control integrin adhesion is further strengthened by our finding that the maintenence of muscle attachments only requires β_{PS}-containing integrins in the muscles. Furthermore, this result demonstrates that these integrins do not have equivalent roles on the two sides of the muscle attachment site. The phenotype of embryonic muscle detachment is observed when α_{PS2} or β_{PS} is missing from the muscles. Conversely, embryos that lack α_{PS1} (Brower et al., 1995) and, as we show here, β_{PS} from the epidermis do not show a detached muscle phenotype. The latter rules out the possibil-

ity that another α_{PS} in the tendon cells complements the loss of α_{PS1}. Since in the absence of β_{PS}, neither α_{PS1} nor α_{PS2} reaches the cell surface (Leptin et al., 1989), this makes it unlikely that another β subunit is able to complement the loss of β_{PS} in the epidermis. Therefore the maintenence of muscle attachments is mediated largely by the presence of $\alpha_{PS2}\beta_{PS}$ in the muscles. This is different from other morphogenetic processes which require these integrins. For example, both integrins are required for the adhesion between the two surfaces of the developing adult wing. The integrin $\alpha_{PS1}\beta_{PS}$ is expressed and required on the dorsal surface, and $\alpha_{PS2}\beta_{PS}$ is expressed and required on the ventral surface (Brabant and Brower, 1993; Brower et al., 1995).

The essential function of $\alpha_{PS2}\beta_{PS}$ in muscle attachment is likely to be composed of two types of attachment: muscle to epidermis and muscle to muscle. The existence of the muscle–muscle attachment is revealed by our examination of the *engrailed, invected* mutant embryos, where in the absence of the appropriate epidermal tendon cells the muscles are still connected end-to-end. Once $\alpha_{PS2}\beta_{PS}$ is localized to the ends of the muscles it could lead to the concentration of ligands to form an extracellular matrix that is required for both muscle–muscle and muscle–epidermis attachment. If the extracellular ligands have two binding sites for $\alpha_{PS2}\beta_{PS}$ then this would account for the muscle–muscle attachment. Presumably additional cell surface receptors are important in the epidermal cells for attachment to the extracellular matrix. Examination of muscle attachment sites by EM shows that there is a large quantity of secreted extracellular matrix between the cells that make up the attachment (Newman and Wright, 1981), so that the best description of muscle attachment might be that both muscles and the tendon cells attach to the extracellular matrix, leading to a connection from muscle to muscle as well as tendon cell to muscle (as diagrammed in Fig. 1).

As the $\alpha_{PS2}\beta_{PS}$ integrin is the first molecule to be identified that marks the ends of the somatic muscles as unique subcellular domains, our experiments are also relevant to the general problem of the role of cell–cell interactions with the epidermis in mesodermal development. Some of the earlier events in muscle development do require signals from the epidermis, such as the specification of the visceral mesoderm by epidermal expression of the TGF-β family member encoded by *decapentaplegic* (*dpp*; Staehling-Hampton et al., 1994; Frasch, 1995).

However, we have shown that the correct pattern of the epidermis is not a prerequisite for the muscles to know that their ends are different. How the polarity arises within the muscles is currently unclear. In the founder cell hypothesis (Bate, 1990; Rushton et al., 1995), each *Drosophila* embryonic muscle arises from a single founder cell, which fuses to surrounding uncommitted myoblasts to generate the muscle. Part of the information imparted to each founder cell during its specification is how many myoblasts will be allowed to fuse to it and where it should make its attachments to the epidermis. During the process of fusion, the muscles do attain their appropriate shape and orientation (Dunin-Borkowski et al., 1995), but fusion is not required, because in mutant embryos where myoblast fusion fails to occur single cells are observed to make the

appropriate attachments to the epidermis (Rushton et al., 1995). These cells are identified as muscle founders by their expression of founder cell–specific markers such as *S59* and *vestigial.* Therefore the polarity of each muscle would seem to arise in its founder cell. It could be that these cells become randomly bipolar, and then become oriented by external cues, or external cues could specify the poles of the founder cells, for example by inducing the formation of a growth cone at particular points on the founder cell surface. Such growth cones are prominant when the muscles are "searching for" the correct attachment sites (Bate, 1990). At least for the longitudinal muscles, the disruption of the pattern of the epidermis does not disrupt these putative external signals, suggesting that they might be provided by other mesodermal cells. Before this work it was not clear whether the specification of the ends of the muscles as specific subcellular domains, as revealed by integrin localization, was a direct consequence of the processes that lead to the formation of muscles with a specific shape and orientation, or whether it was a result of secondary integrin-dependent interactions between the muscles and the tendon cells. Our results show that integrin localization does not require interaction of the muscles with the epidermis nor integrin function, but we expect that we will in future identify molecules that are localized to the ends of the muscles in response to integrin function.

Possible Mechanisms for the Internally Directed Localization of $\alpha_{PS2}\beta_{PS}$

We have shown that when we substitute the cytoplasmic tail of a heterologous transmembrane protein for the cytoplasmic tail of the β_{PS} subunit, then this chimeric protein becomes localized to the ends of the muscles in the *Drosophila* embryo. There are two general mechanisms that could account for CD2/β_{PS} localization. (*1*) The proteins could be inserted randomly into the plasma membrane and subsequently become localized to the ends of the muscles, either by lateral diffusion in the membrane and capture by localized intracellular proteins, or by directed movement in the plane of the membrane, for example by kinesin driven movement along cortical microtubules. Alternatively, (*2*) the proteins could be inserted locally into the plasma membrane at the ends of the muscle by vesicle targeting. We cannot currently distinguish between these mechanisms, but all are dependent on a polarized cytoskeleton. A third possible mechanism, mRNA localization, has been discounted by two results. First the β_{PS} mRNA is not found localized to the ends of the muscle when visualized by in situ hybridization (our unpublished observations), and second one can observe some intracellular accumulation of the CD2/β_{PS} chimeric proteins (particularly C$\beta\beta$) throughout the muscle, which is probably newly synthesized protein that is slowly being folded. Thus the protein appears to be synthesized throughout the cell.

If $\alpha_{PS2}\beta_{PS}$ is inserted randomly into the plasma membrane and becomes localized by the β_{PS} cytoplasmic tail binding to cytoplasmic proteins, then one question that arises from studies on focal adhesions is whether the β_{PS} cytoplasmic tail would be able to bind to these cytoplasmic proteins before ligand binding. In cell culture a wild-type

integrin must be bound to a ligand for it to become associated with a focal adhesion formed by another integrin bound to its ligand (Fath et al., 1989; LaFlamme et al., 1992). The requirement for extracellular ligand binding is lost when the β cytoplasmic tail is fused to a heterologous monomeric transmembrane protein (Fath et al., 1989; Geiger et al., 1992; LaFlamme et al., 1992) or when the α cytoplasmic tail is deleted from an integrin heterodimer (Briesewitz et al., 1993; Ylanne et al., 1993). Thus binding of integrins to extracellular ligands results in a conformational change that allows the association of the β cytoplasmic tail with one or more cytoplasmic components of the focal adhesion. This change, which may be a conformational change in the β cytoplasmic tail or a displacement of an inhibitory α cytoplasmic tail, is mimicked by the fusion of a β cytoplasmic tail to a heterologous protein or the deletion of the α cytoplasmic tail. If the localization of $\alpha_{PS2}\beta_{PS}$ to the ends of the muscles is similar to the localization of an integrin to a focal adhesion, then $\alpha_{PS2}\beta_{PS}$ would have to bind to an extracellular ligand before the β_{PS} cytoplasmic tail would be able to bind to the cytoplasmic components that direct its localization. Thus $\alpha_{PS2}\beta_{PS}$ could bind to soluble extracellular ligands all over the surface of the muscle, and then become localized to the muscle termini. Alternatively, since some cytoplasmic proteins, such as focal adhesion kinase, can associate with integrins before extracellular ligand binding (Miyamoto et al., 1995), the $\alpha_{PS2}\beta_{PS}$ integrin could become localized first, and then bind to extracellular ligands. We need to identify the cytoplasmic proteins that interact with the β_{PS} tail and result in its localization to resolve this question. Since in either case the localization of the $\alpha_{PS2}\beta_{PS}$ integrin will lead to the localization of ligands at the muscle termini, this open question does not alter the potential significance of the intracellular localization of integrins in the developing embryo.

In summary, although we have yet to completely rule out the outside-in model, our results demonstrate that inside-out localization of integrins occurs, and may be sufficient to account for integrin function in the developing muscles. In the inside-out model, the $\alpha_{PS2}\beta_{PS}$ integrin is initially localized by the muscle cell cytoplasm to the ends of the muscles. The localized integrin can then organize the specialized attachments. Diffusible extracellular $\alpha_{PS2}\beta_{PS}$ ligands would accumulate between the ends of the muscles by binding to $\alpha_{PS2}\beta_{PS}$ and mediate muscle–muscle and muscle–epidermis attachment. Once the integrins are localized and bound to ligands, they could then recruit additional cytoskeletal proteins leading to a more elaborate attachment of the cytoskeleton to the membrane.

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